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**14. ABSTRACT**

Blast trauma causes onset and chronic tinnitus and hearing impairment in rats. The induced chronic tinnitus latter shifts to the high-frequency region. Blast also induces onset hyperactivity in the auditory brainstem, which later shifts to the auditory cortex. The induced tinnitus is accompanied by increased neuronal activity in limbic structures such as the amygdala and anterior cinculate cortex and by increased anxiety. Compared to concussion, blast exposure induces significant TBI as revealed by sustained astrogliosis and axonal injury in auditory brain centers. In patients with blast induced hearing loss and tinnitus, we show significant relations among hearing, psychometric, neuropsychological variables and neuroimaging related parameters; most notably metabolites and neurotransmitters and white matter tracks vis-à-vis the FA metric. Patients with blast-induced hearing loss and tinnitus had slowed reaction time, increased reaction time variability, and lower accuracy that may be associated with difficulty inhibiting attention to distracting tinnitus signals. Several aspects of processing speed and delayed incidental are associated with tinnitus severity.

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## INTRODUCTION:

Blast- or concussion-induced traumatic brain injury (TBI) is often associated with tinnitus which is a perception of bothersome sound in the absence of external stimulation. The goal of this project is to determine that blast- and concussion-induced tinnitus results from a cascade of plastic changes in auditory brain structures. The scope of the research effort will cover investigations using both animal and human models. During the third year, we continued the planned experimentation on both animal and human models. In animal studies, we conducted more experiments, analyzed the electrophysiological and MEMRI data, and finalized and submitted manuscripts. We accomplished our goals of the first two phases concerning blast-induced tinnitus and traumatic brain injury (TBI) and concussion-induced tinnitus and TBI. At the same time, we have transitioned to the final phase, which is to investigate combined blast-and concussion-induced tinnitus and related TBI. In human studies, we performed further data analysis and have established trends in various imaging procedures in humans (diffusion-tensor imaging, DTI; susceptibility-weighted imaging, SWI; and MR-spectroscopy, MRS), and their relationship to audiometric (hearing loss), psychophysical (word recognition in quiet and noise, loudness), psychometric (response to questionnaires), and neuropsychological data in those subjects with blast induced hearing loss and tinnitus.

## BODY OF REPORT:

### REVIEW OF ANIMAL STUDIES:

### A. BLAST-INDUCED TINNITUS AND HEARING LOSS

#### 1. Blast-induced tinnitus and hearing loss

Behavioral and auditory brainstem response (ABR) tests were conducted before and after blast exposure. Gap-detection (GAP) and pre-pulse inhibition (PPI) testing were used to assess behavioral evidence of tinnitus and hearing status. As described in previous studies (Turner et al., 2006, Yang et al., 2007, Luo et al., 2012, Pace and Zhang, 2013), 2 kHz band noise of 6-8, 10-12, 14-16, 18-20, 26-28 kHz and broadband noise (BBN, 2-30 kHz range) was presented at 60 dB SPL to rats as background noise. For each band frequency, a total of 16 trials consisted of eight startle only and eight trials with a 40 ms silent gap prior to a broad-band noise (50 ms, 115 dB) as a startle stimulus. PPI procedure and parameters were similar to GAP except that no background noise or silent gaps were used. A pre-pulse (50 ms, 60 dB SPL) was introduced 100 ms before the startle stimulus. The rat reduced its acoustic startle reflex in response to the pre-pulse, except when there was hearing loss at a frequency similar to the pre-pulse. For the groups that were monitored one and three months after blast exposure, behavior and ABR testes were routinely conducted until completion of electrophysiological recordings.

**Figure 1**

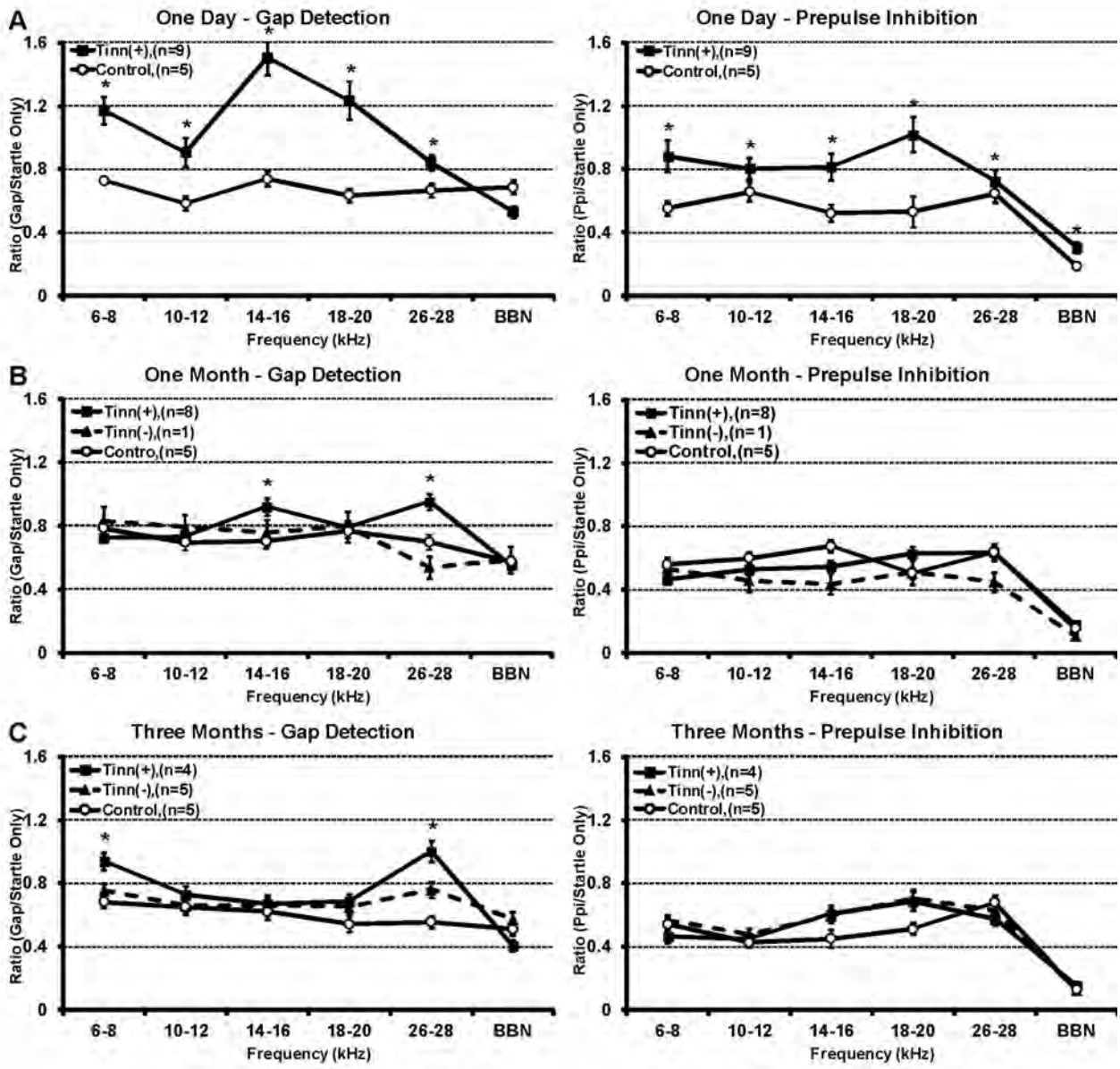



Figure 1. Gap detection ratio values (gap detection/Startle-only response) and PPI ratio values (PPI/startle-only response) measured from tinnitus positive animals, tinnitus negative animals and age-matched control animals, at one day after blast exposure (A), one month after blast exposure (B) and three months after blast exposure (C). Note that rats showed significant deficits in gap-detection and PPI inhibition at one day after blast exposure, followed by marked tinnitus at one month after blast exposure on gap detection test. Further tinnitus frequency spread was found at three months after blast exposure on gap detection test. Error bars represent standard error of the mean. * $p<0.05$.

## Figure 2

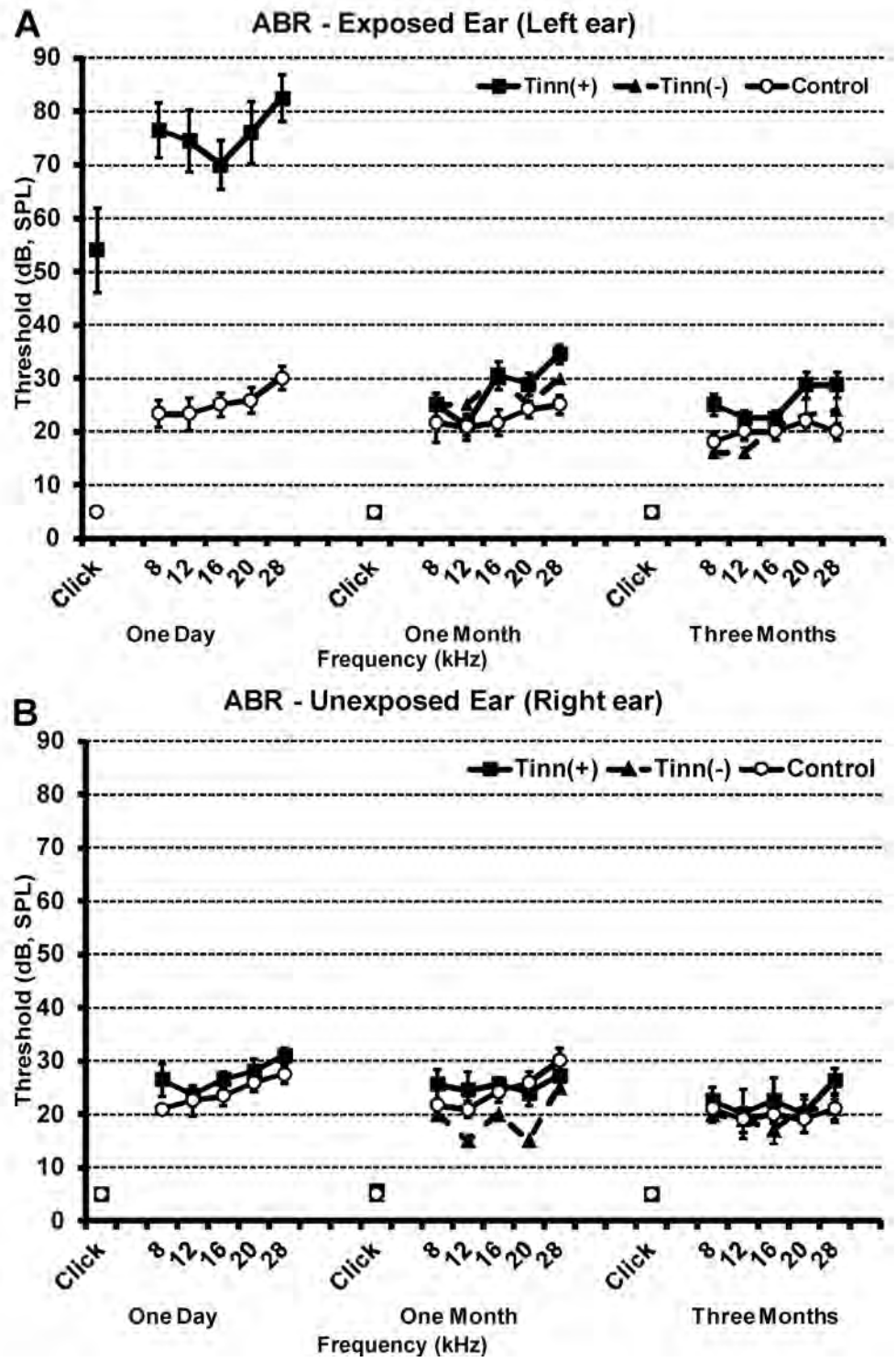



Figure 2. ABR thresholds were measured in the left and right ears at one day, one month and three months after blast exposure. ABR thresholds of the tinnitus positive animals were significant elevated at one day after blast exposure, and then returned to normal level at one month and three months after blast exposure. Error bars represent standard error of the mean.

## Figure 3

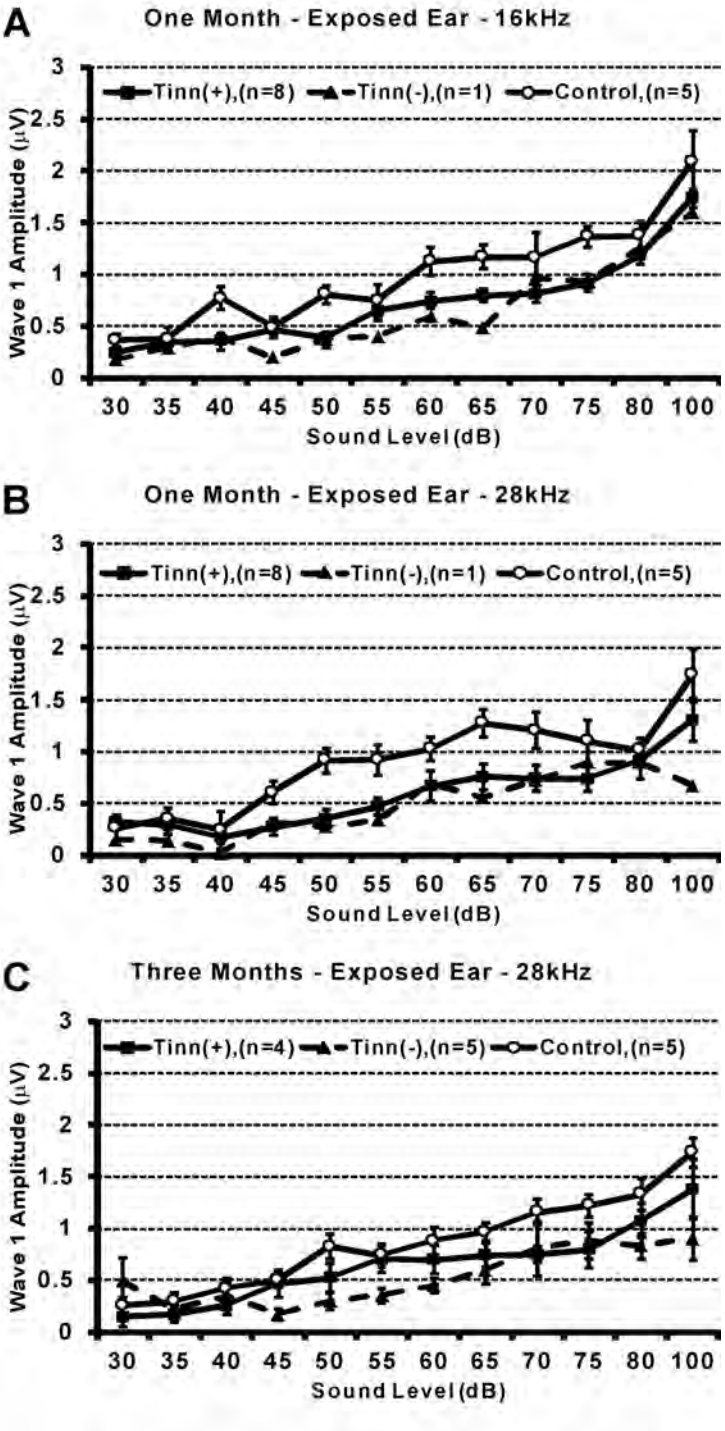



Figure 3. P1-N1 amplitudes of ABRs were measured at left and right ears at one day, one month and three months after blast exposure. The P1-N1 amplitudes were measured at 14-16 kHz and 26-28 kHz of one month after blast exposure, at 26-28 kHz of three months after blast exposure. Note the significant degradation of P1-N1 amplitudes. Error bars represent standard error of the mean.

*One day after blast exposure*

Behaviorally, as indicated by significant increase in GAP ratio values, the blasted rats showed significantly impaired gap detection compared to non-blasted controls. As can be seen in Figure 1A, GAP ratio values from blasted rats were significantly higher at all frequencies including 6-8 kHz ($F_{(1,127)}$=6.966, $p$<0.01), 10-12 kHz ($F_{(1,125)}$=5.522, $p$<0.01), 14-16 kHz ($F_{(1,127)}$=5.032, $p$<0.01), 18-20 kHz ($F_{(1,127)}$=4.6, $p$<0.05) and 26-28 kHz ($F_{(1,126)}$=2.011, $p$<0.05). Significantly increased PPI ratio values were also found in blast-exposed rats at 6-8 kHz ($F_{(1,123)}$=4.302, $p$<0.05), 10-12 kHz ($F_{(1,127)}$=4.423, $p$<0.05), 14-16 kHz ($F_{(1,126)}$=6.175, $p$<0.01), 18-20 kHz ($F_{(1,123)}$=3.970, $p$<0.05), 26-28 kHz ($F_{(1,123)}$=5.864, $p$<0.05) and broadband noise ($F_{(1,124)}$=4.015, $p$<0.05) (one-way ANOVA test and *post-hoc* Bonferroni tests, Fig. 1A). The significant increase in GAP ratio values indicated strong evidence of tinnitus at these frequencies and the increase in PPI ratio values indicated compromised auditory detection.

To further examine the impact of blast exposure on hearing, we measured changes in ABR thresholds. As shown in Figure 2, blast exposure induced significant threshold shifts compared to controls in response to both click and tone burst stimuli, with an average threshold shift of approximately 50 dB SPL. The thresholds of unexposed right ears of blasted rats showed no change compared to the non-blasted controls.

*One month after blast exposure*

Behaviorally, one month after blast exposure, our results showed that there were eight tinnitus positive and one tinnitus negative rats. As can be seen in Figure 1B, those tinnitus positive rats had significant increase in GAP ratio values at 14-16 kHz ($F_{(1,127)}$=4.29, $p$=0.037) and 26-28 kHz ($F_{(1,127)}$=10.59, $p$=0.001) (one-way ANOVA and *post-hoc* Bonferroni tests, Fig. 1B). The results indicated that the induced tinnitus tended to be in the middle and high frequency regions. However, the PPI ratio data from tinnitus positive rats didn't show significant impairments at any frequencies compared to the control group (Fig. 1B). This suggests that the comprised GAP detection responses specifically reflected behavioral evidence of tinnitus.

When examining the impact of blast exposure on hearing threshold, our ABR results showed that the thresholds of the exposed ear, while significantly elevated at 1-day post-blast, returned to control group levels by one month post-blast. The unexposed ears of blasted rats showed no difference compared to the control rats (Fig. 2). Since it has been shown that temporary threshold shift recovery is necessarily indicative of hearing recovery (Kujawa and Liberman, 2009), we further examined the impact of blast exposure on these rats by measuring the changes in P1-N1 amplitudes. The results from tinnitus positive rats demonstrated significant degradation in the P1-N1 amplitudes at 14-16 kHz ($F_{(2,165)}$=5.745, $p$<0.01; Fig. 3) and 26-28 kHz ($F_{(2,166)}$=8.731, $p$<0.01; Fig. 3), indicating that blast-induced damage to the auditory system persisted after blast exposure.

*Three months after blast exposure*

Three months after blast exposure, four rats continued to manifest behavioral evidence of tinnitus while five rats no longer exhibited tinnitus, the latter of which were placed in the tinnitus negative group. Compared to the sham controls, the GAP ratio values of tinnitus positive rats were significantly increased at 6-8 kHz ($F_{(1,127)}$=3.89, $p$<0.05) and 26-28 kHz ($F_{(1,127)}$=6.84, $p$<0.01) (One-way ANOVA test, Fig. 1C). The results appeared to be consistent with the fact that the induced tinnitus occurred at both low and high frequency regions. PPI data didn't show any significant differences at any frequency bands, again suggesting that gap-detection data was not influenced by compromised hearing detection (Fig. 1C).

Consistent with PPI data, ABR data showed no significant differences in hearing thresholds in the blasted left ears and non-blasted right ears compared to controls (Fig. 2). We further examined the impact of blast exposure on hearing by measuring the P1-N1 amplitude at 6-8 kHz and 26-28 kHz of blasted ears of tinnitus positive and negative rats. We found that the P1-N1 amplitude was significantly decreased at 26-28 kHz ($F_{(2,166)}$=8.738, $p$<0.01; Fig. 3) and no

significant difference at 6-8 kHz. This indicated that animals' hearing was still significantly compromised at high-frequencies, even though the threshold recovered.

**2. Neural activity changes in the dorsal cochlear nucleus of rats with blast-induced tinnitus**

Electrophysiological recordings were performed for all animal groups. Briefly, each rat was first anesthetized with a mixture of air (1 l/min) and isoflurane (5% v/v). The surgical area on its head was then shaven and cleaned. Once no reflex was observed when pinching the hind paw, the rat was placed on a stereotaxic apparatus (Kopf Model 1530) with a pair of custom-made hollow ear bars for sound stimulation. A mixture of air (1 l/min) and isoflurane (1.75-2.5% v/v) was used to keep the animal unconscious during surgery and recordings. A thermostat-controlled blanket (Harvard Apparatus) was used throughout the procedure to maintain the animal's body temperature at 37 $^{o}$C. Craniotomy was performed to expose the DCN. Briefly, a small piece of occipital bone and underlying dura mater above the left DCN were removed. Following partial aspiration of the cerebellar tissue overhanging the left DCN, its dorsal view was revealed. Using a micromanipulator (Kopf Model 1460-61), a 32-channel electrode probe (NeuroNexus Technologies, Inc.) was inserted into the DCN. The probe had 8 shanks separated by 200 µm (all distances are center to center) and each shank had four recording sites linearly spaced at 50 µm intervals. Each shank was 15 µm thick, 2 mm long, and tapered in width from 33 µm to a few microns at the tip. Prior to insertion, the electrode probe was dipped into a 3% DiI solution (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen) prepared with dimethylformamide to label electrode insertion tracks. The probe was inserted to a depth of 150-200 µm below the surface of the DCN, corresponding to the fusiform cell layer (Waller and Godfrey, 1994). After probe placement, the brain was covered with agarose to avoid tissue swelling and drying. Spike activity was acquired using a TDT-system 3 (RZ2) data acquisition system. Neural signals were sampled at 25 kHz and filtered at 100-3000 Hz, with threshold set to 1.5 times the standard deviation. Spontaneous single- and multi-unit spikes were recorded twice; one 5 min prior to and one 5 min after performing frequency tuning curve (FTC) construction. Each spontaneous recording period lasted 5 minutes. We used the spontaneous activity data after FTC measures, since they were more stable than those collected before FTC measures. The latter may be due to closer proximity in time to surgical effects.

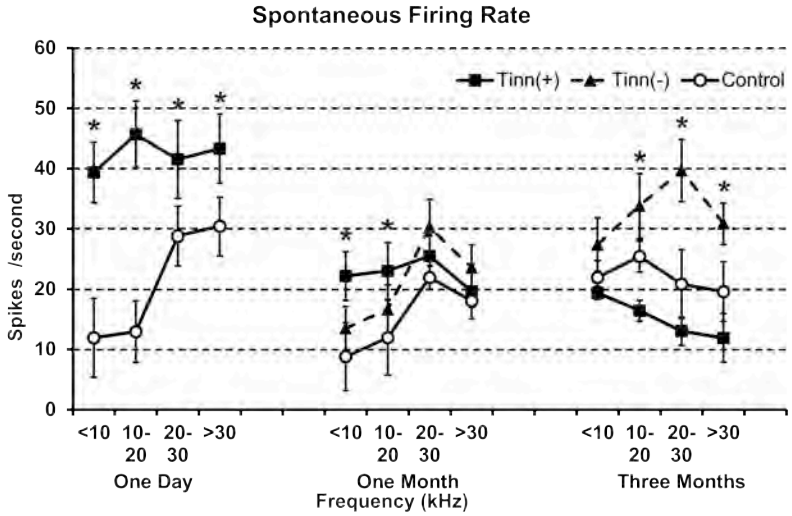



Figure 4. Based on the characteristic frequency recorded at four frequency bands (<10 kHz, 10-20 kHz, 20-30 kHz and >30 kHz), SFR was measured from tinnitus positive animals, tinnitus negative animals and age-matched control animals, at one day, one month, and three months after blast exposure. Error bars represent standard error of the mean. * $p$<0.05.

*One day after blast exposure*

As shown in Figure 4, we found a significant increase in spontaneous firing rate across the entire mediolateral span of the DCN one day after blast exposure. Specifically, as can be seen in Figure 3A, spontaneous firing rate in blasted rats was increased 1.2 times in the middle-to-high and high frequency regions and increased 1.6 times in the low and low-to-middle frequency regions, compared to the control group. The increase in spontaneous firing rates in blasted rats was statistically significant at four frequency bands spanning a tonotopic range of 2-42 kHz in the DCN: <10 kHz ($F_{(1,110)}$=11.625, $p$<0.01), 10-20 kHz ($F_{(1,111)}$=12.011, $p$<0.01), 20-30 kHz ($F_{(1,108)}$=4.430, $p$<0.05), >30 kHz ($F_{(1,107)}$=4.736, $p$<0.05, one-way ANOVA test and *post-hoc* Bonferroni tests).

*One month after blast exposure*

We found that spontaneous firing rates at one month after blast exposure were also increased, albeit to a smaller degree compared to one day after exposure. Compared to the control group, increased SFRs were found in both tinnitus positive and tinnitus negative groups and were broadly distributed across the entire mediolateral expansion of the DCN. This increase, however, differed from that seen at one day after blast exposure. First, the tinnitus positive rats showed significantly higher SFRs at the <10 kHz and 10-20 kHz regions, but tinnitus negative rats did not show significant difference. The SFRs ratio increased 2.5 times at the <10 kHz region and 1.9 times at the 10-20 kHz region. Second, the tinnitus negative rat showed relatively uniform SFR increases across the four frequency regions, but the increase was slightly higher (1.5 times) in the >30 kHz region than the <10 kHz region (1.3 times) (Fig. 4).

*Three months after blast exposure*

As can be seen in Figure 4, the patterns of activity changes three months after blast exposure were quite different from those found at one day and one month after blast exposure. Specifically, while tinnitus positive rats showed an increase in spontaneous firing rates at one day and one month after blast exposure, at three months after blast exposure, they showed decreased SFRs across the entire mediolateral expansion of the DCN. This decrease reached 0.6 times at the >30 kHz, 20-30 kHz and 10-20 kHz regions and 0.9 times in the <10 kHz region. On the contrary, tinnitus negative rats showed increased SFRs across all frequency bands, which became comparable to that seen in the tinnitus negative group at one month after blast exposure. Specially, the increases were 1.6 fold in the 20-30 kHz and >30 kHz regions compared to the sham control group (Fig. 4). The results were statistically significant at the 10-20 kHz ($F_{(2,102)}$=5.385, $p$<0.01), 20-30 kHz ($F_{(2,104)}$=14.156, $p$<0.01) and >30 kHz ($F_{(2,108)}$=8.672, $p$<0.01).

**3. Neural activity changes in the inferior colliculus of rats with blast-induced tinnitus**

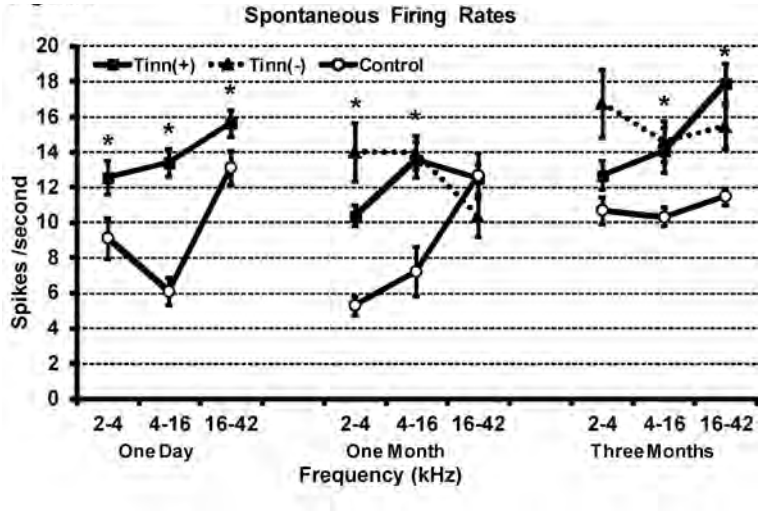


Figure 5. SFRs recorded in three frequency regions (2-4 kHz, 4-16 kHz and 16-42 kHz) in the IC of tinnitus positive, tinnitus negative and age-matched control rats at one day, one month, and three months after blast exposure. Note the significant increase in SFRs in all frequencies regions at one day after blast exposure, in the 2-4 kHz and 4-16 kHz regions at one month after blast exposure, and in the 4-16 kHz and 16-42 kHz regions at three months after blast exposure. Error bars represent standard error of the mean. * $p$<0.05.

Electrophysiologically, we also investigated neural activity changes in the inferior colliculus (IC). At one day after blast exposure, we found a significant elevation in SFRs across the entire dorsoventral span of the right IC, contralateral to the unplugged, blast-exposed left ear. Specifically, as can be seen in Figure 5, SFRs in tinnitus positive rats increased 1.4 times in the low frequency region, 2.2 times in the middle frequency region, and 1.2 times in the high frequency region compared to the control group. These increases were statistically significant at 2-4 kHz ($F_{(1, 93)}$ =11.4.81, p<0.05), 4-16 kHz ($F_{(1, 157)}$ =33.011, p<0.01), and 16-42 kHz ($F_{(1, 180)}$ =4.030, p<0.05) (one-way ANOVA with post hoc Bonferroni tests). At one month after blast exposure, we found increased SFRs in both tinnitus positive and negative groups in low and middle frequency regions, compared to the control group. This increase, however, differed from that seen at one day after blast exposure. First, tinnitus positive rats showed significantly higher SFRs in the 2-4 kHz and 4-16 kHz regions, with increases of 1.96 and 1.9 times, respectively. The activity rate in the 16-42 kHz region was no longer significantly increased. Second, tinnitus negative rats showed relatively uniform increase in SFRs (14 spikes/s) in both the low and middle frequency regions, but a slightly higher increase (2.65 times) in the 2-4 kHz region, compared to the control group. These increases were statistically significant in the 2-4 kHz ($F_{(2, 80)}$ =14.738, p<0.01) and 4-16 kHz regions ($F_{(2, 134)}$ =6.427, p<0.01). We also found that the patterns of activity changes at three months after blast exposure were quite different from those found at one day and one month after exposure. The SFRs of three months after blast exposure showed an increase in tinnitus positive and tinnitus negative groups across all frequency regions, with significant increases at 4-16 kHz ($F_{(2, 157)}$ =5.869, p<0.01) and 16-42 kHz ($F_{(2, 181)}$ =5.498, p<0.01).

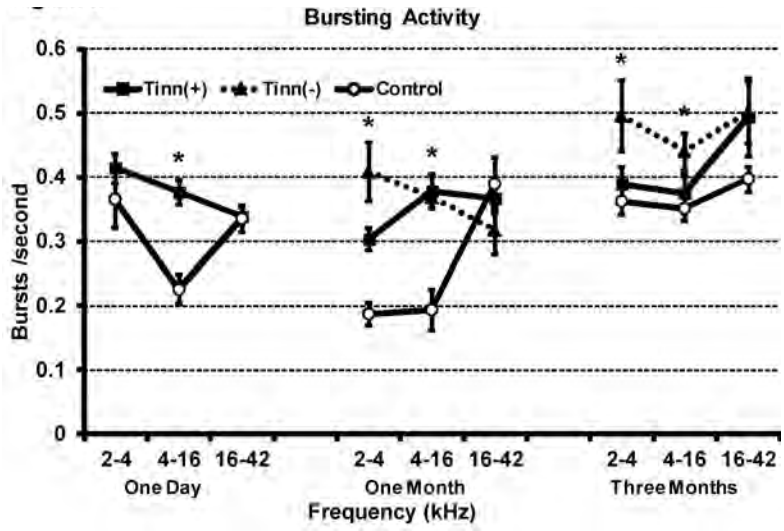



Figure 6. Bursting activities measured in three frequency regions (2-4 kHz, 4- 16 kHz and 16-42 kHz) in the IC of tinnitus positive, tinnitus negative, and age-matched control rats at one day, one month, and three months after blast exposure. Note the significant elevation in bursting rates in the 4-16 kHz region at one day after blast exposure and in the 2-4 kHz and 4-16 kHz regions at one month and three months after blast exposure. Error bars represent stand error of the mean. * $p$<0.05.

As can be seen in Figure 6, bursting rate significantly increased (1.7) times at the 4-16 kHz frequency region ($F_{(1, 157)}$ =15.920, p<0.01) at one day after blast exposure (one-way ANOVA with post hoc Bonferroni tests). At one month after exposure, bursting activity increased in the 2-4 kHz and 4-16 kHz regions for both tinnitus positive and tinnitus negative groups. The increases were statistically significant in the 2-4 kHz ($F_{(2,80)}$ =9.98, p<0.01) and 4-16 kHz ($F_{(2, 134)}$ =8.621, p<0.01) regions. At three months after blast exposure, both tinnitus positive and tinnitus negative groups showed increased bursting rates at all frequency regions. However, significance was reached only in the 2-4 kHz ($F_{(2, 90)}$ =3.310, P<0.05) and 4-16 kHz regions ($F_{(2, 159)}$ =3.372, p<0.05). There is no significant difference in the increased bursting rates between tinnitus positive rats and control rats.

**4. Neural activity changes in the auditory cortex of rats with blast-induced tinnitus**

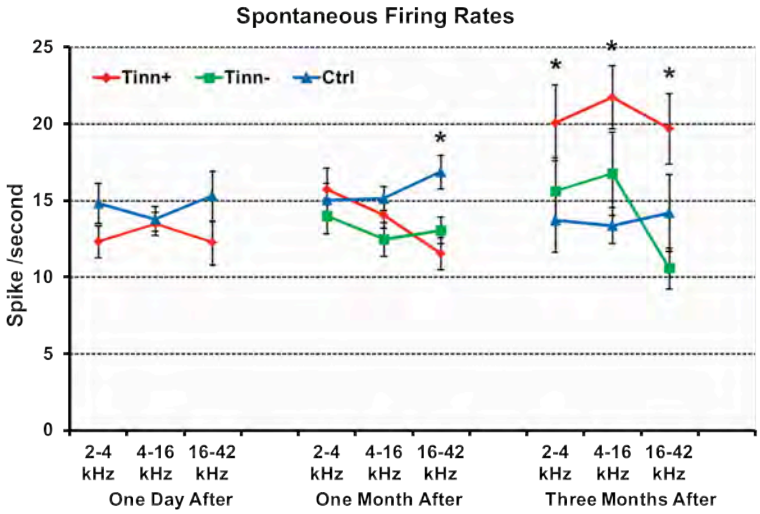



Figure 7. SFRs recorded in three frequency regions (2-4 kHz, 4-16 kHz and 16-42 kHz) in the AC of tinnitus positive, tinnitus negative and age-matched control rats at one day, one month, and three months after blast exposure. Note the significant increase in SFRs in the high frequency regions at one and three months after blast exposure. Error bars represent standard error of the mean. * $p$<0.05.

In addition to recording from the DCN, IC, we recorded from the auditory cortex (AC). This allowed use to understand how neural activity changed along the auditory pathways. As shown in Figure 7, at one day after blast exposure, there was no significant difference between tinnitus positive rats and control rats in the SFR of all three frequency regions, and also there was no difference in the burst activity, which indicates the auditory cortex may not involve in the tinnitus generation one day after blast exposure. From the one month after blast exposure group, the SFR was significant lower of tinnitus positive rats at high frequency region (F (2, 51) =5.264, p<0.05), there was no significant difference at low frequency region and medial frequency region; however, the results of bursting activity showed significant higher of the tinnitus positive rats at medial frequency region, that suggested blast exposure may cause neural activity of the auditory cortex start at one month after blast exposure. In the three months after blast exposure, significant higher of the SFRs were found at all three frequency regions of tinnitus positive group (low frequency region: F(2, 55) =3.789, p<0.05; medial frequency region: F (2, 167) =6.110, p<0.05; high frequency region: F (2, 53) =6.898, p<0.05), special in the medial frequency region, the SFR of tinnitus positive rats were 1.6 times of control groups.

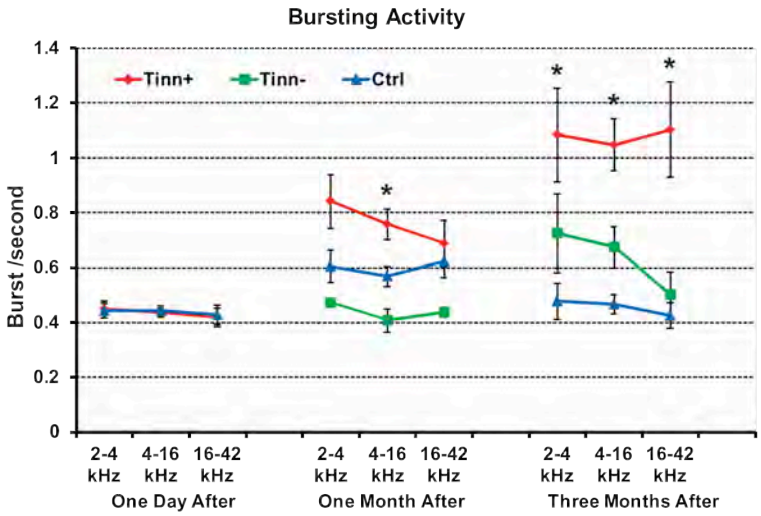



Figure 8. Bursting activities measured in three frequency regions (2-4 kHz, 4- 16 kHz and 16-42 kHz) in the AC of tinnitus positive, tinnitus negative, and age-matched control rats at one day, one month, and three months after blast exposure. Note the significant elevation in bursting rates in the 4-16 kHz region at month after blast exposure and in all the frequency regions at three months after blast exposure. Error bars represent stand error of the mean. * $p<0.05$.

As shown in Figure 8, for the bursting activity, the significant elevation of the burst rates of tinnitus positive rats were also found at low frequency region ($F(2, 55) = 5.206$, $p<0.05$), medial frequency region ($F(2, 167) = 16.679$, $p<0.05$) and high frequency region ($F(2, 53) = 11.718$, $p<0.05$), that indicated the blast exposure may cause robust neural activity changes at the auditory cortex three months after blast exposure.

**5. Manganese-enhanced MRI (MEMRI) imaging**

Blast-induced tinnitus is a common health condition among soldiers and veterans who experience blast-related trauma. These tinnitus sufferers frequently experience deficits in limbicassociated functioning such as anxiety, memory loss, and depression. It has been suggested that there is strong limbic involvement in the etiology of noise-induced tinnitus, however, it remains unclear how blast invokes the limbic system in tinnitus-related emotional and cognitive problems. In this study, rats were blast-exposed and tested behaviorally for tinnitus, anxiety and spatial cognition using gap detection acoustic startle reflex testing, elevated plus maze and Morris water maze, respectively. Blast-induced neural activity changes in several limbic and paralimbic structures were evaluated using manganese-enhanced magnetic resonance imaging (MEMRI).

Five weeks after the blast exposure, all rats were scanned with a 7.0 T Siemens ClinScan MRI scanner (Siemens Medical Solutions USA, Inc. Malvern, PA). Rats were injected with MnCl2 (67 mg/kg body weight) intraperitoneally. After the injection, rats were placed in a soundproof room for 8 hours to allow absorption and neuronal uptake of manganese. Accumulative uptake of manganese for 8 hours is adequate for functional imaging, and utilization of anesthesia during scanning does not affect manganese uptake (Lee et al., 2005, Bissig and Berkowitz, 2009). Images were processed with R (v2.12.1, http://www.r-project.org) scripts developed inhouse by Dr. D. Bissig. The addition of two sets of MPRAGE images was divided by the addition of two sets of PDGE images to mitigate the intensity field bias. The corrected images were used for analysis. Regions of interest (ROIs) were manually drawn using MRIcro v.1.40 with gingerly

referencing the Paxinos and Watson (4th Edition; 1998) rat brain atlas (Figure 9). Average signal intensity of ROIs was obtained with MRIcro. ROI signal intensities (SI) were normalized with that of adjacent noise (SNR = 1000*SI_ROI/SI_noise). Signal-to-noise normalization has been utilized to study auditory structures with MEMRI.

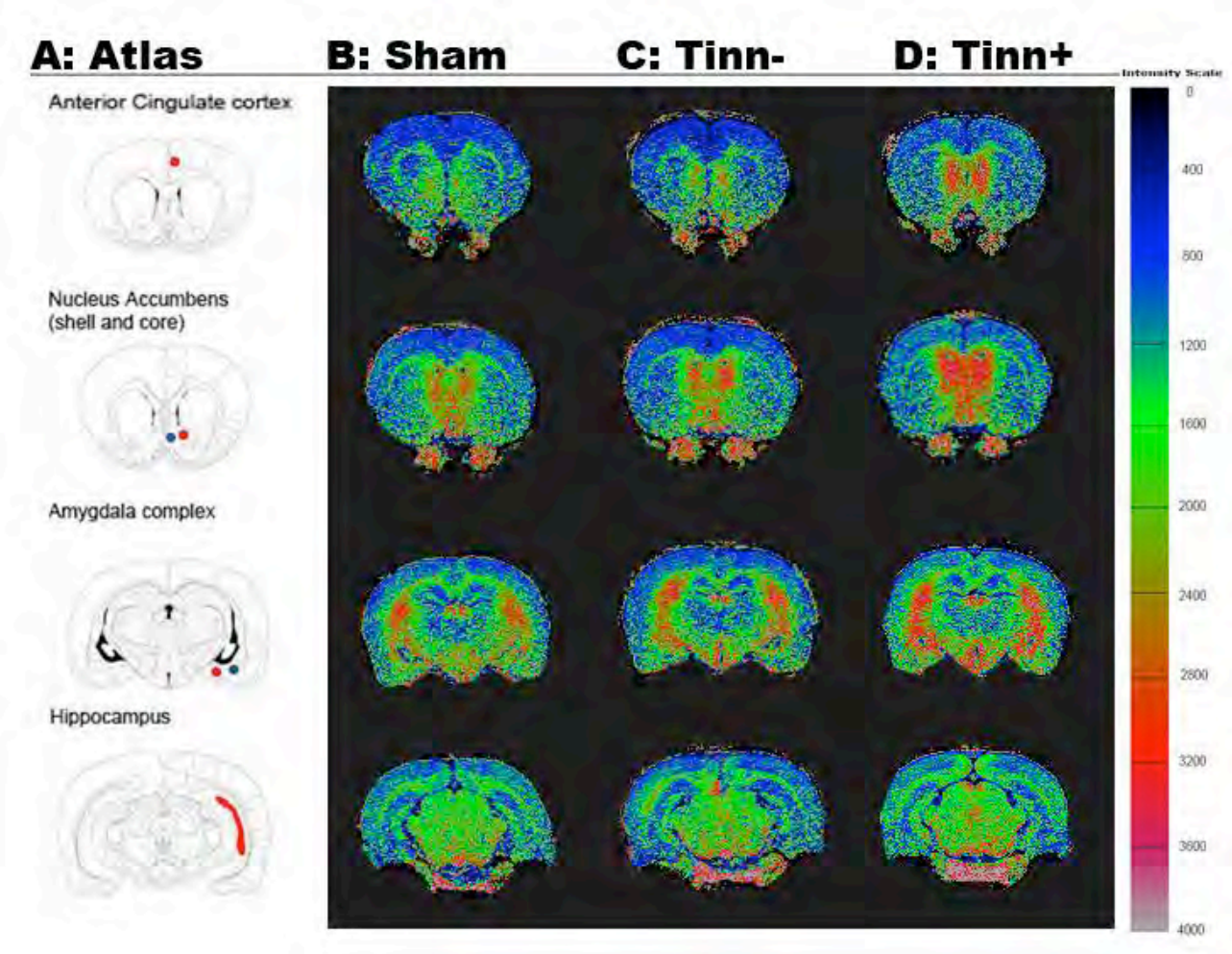



Figure 9. The illustration of regions of interest (ROI). Column A: Rat brain atlas. From top to bottom: anterior cingulate cortex (red dot), nucleus accumbens (red dot is core, and blue dot is shell), amygdala (red dot is superficial group, and blue dot is deep group), and hippocampus (red line is CA1/CA2 regions). Column B through D are representative images from the sham, tinnitus negative, and tinnitus positive groups.

*Measurement of SNR in the hippocampus (Figure 10)*. Intensity of hippocampus was obtained with ImageJ. Three consecutive slices were used on each side. The first slice was caudal to the slice where the dentate gyrus became evident. Four lines were drawn on each side so that 1) they were perpendicular to the curvature of the forceps of the major corpus callosum; 2) the midpoint of the line aligned with the border of the forceps major, and; 3) the direction of the line was toward the center of the parenchyma (Figure 12A). The lines were drawn on the PDGE image set then copied to the MPRAGE image set on the same corresponding coronal slice. A gray value linear plot was generated from each line copied to the MPRAGE image (Figure 10B).

The x-axis represents the distance the line “travels”, and y-axis represents the signal intensity along the line in the form of a “gray value” The peak whose distance just passed mid-point represented the position of hippocampal CA1 and CA2 neurons. The sum of the peak values on one side of the hemisphere was used to represent signal intensity of each hippocampal ROI.

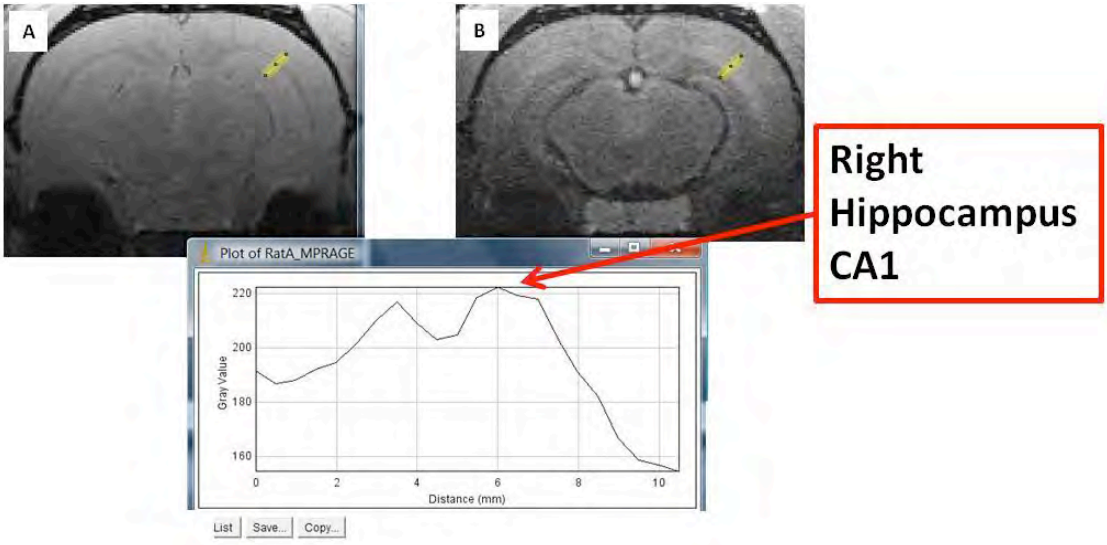



Figure 10. Illustration of the method to obtain signal intensity values for CA1/CA2 regions of the hippocampus. A: picture of a PDGE image. The yellow line indicates the ROI. B: ROI is copied to MPRAGE image.

*Gap detection and prepulse inhibition acoustic startle reflex testing for tinnitus and hearing impairment*

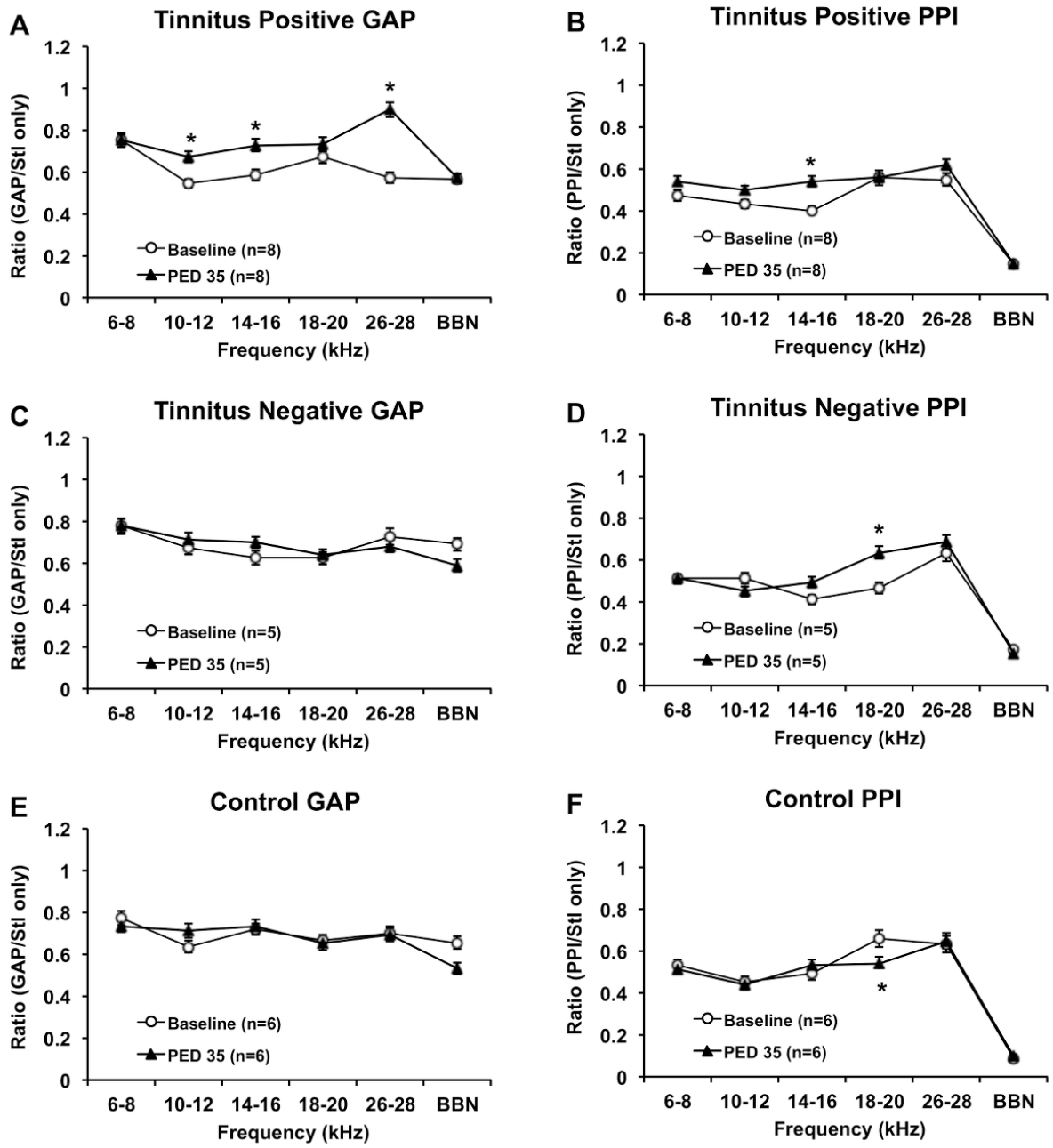



Figure 11. GAP and PPI ratios for the tinnitus positive, tinnitus negative and sham groups. Gap detection data revealed that the tinnitus positive group exhibited robust behavioral evidence of 12, 16, and 28 kHz tinnitus at 35 days after exposure (PED35) compared to baseline (BL) [significant increase in ratio ($p<0.05$) (A)], while the tinnitus negative and sham groups exhibited no evidence of tinnitus [no significant increase in ratio ($p>0.05$), (C)&(E)]. Prepulse inhibition data showed auditory detection deficits at 35 days after blast exposure at 16 kHz in the tinnitus positive group ($p<0.05$) (B); (2) at 20 kHz in the tinnitus negative group ($p<0.05$) (D) but a significant decrease in PPI ratio at 20 kHz ($p<0.05$) (F)]. Error bars represent SEM.

As described in the above section, behavioral testing for tinnitus was conducted using gap detection and prepulse inhibition acoustic startle reflex paradigm. As can be seen in Figure 11, blasted rats with elevated gap-detection ratios and unaffected PPI ratios prior to MEMRI scanning were placed into the tinnitus positive group while the others were placed into the tinnitus negative group. As can be seen in Figure 11 (A &B), the tinnitus positive group ("Tinn+", n=8, Figure 11A & B) exhibited robust evidence of tinnitus at 28 kHz five weeks after blast exposure ($p < 0.05$) whereas the tinnitus negative group ("Tinn-", n=5, Figure 11C & D) and the sham group ("Sham", n=6, Figure 11E & F) did not ($p > 0.05$).

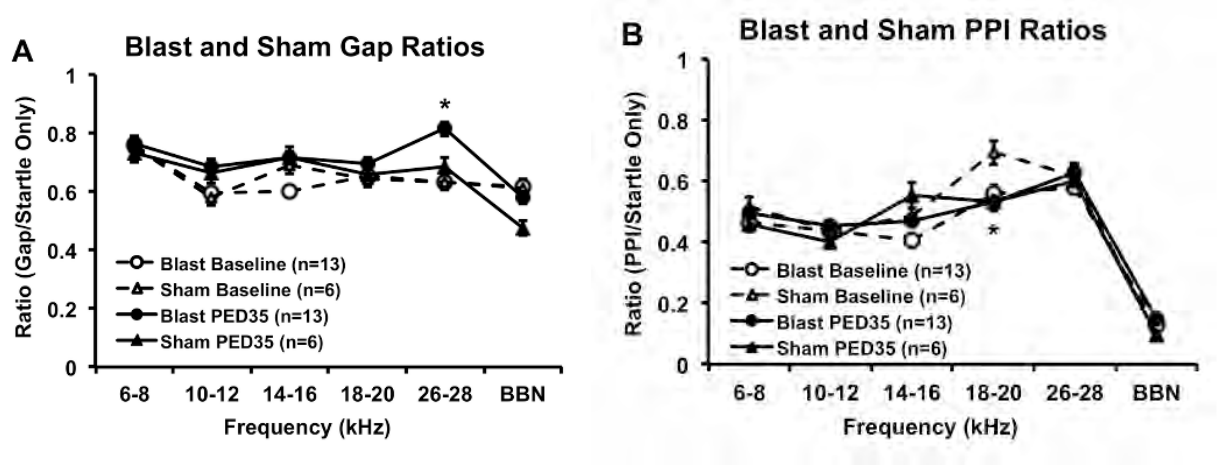



Figure 12. GAP and PPI ratios for the blast and sham groups. Gap detection data showed that (1) 35 days after the blast exposure (PED35), blast group (n=13) exhibited strong behavioral evidence of tinnitus (BL) at 28 kHz [significant increase in ratio ($p<0.05$), (A)]; and (2) 35 days after blast exposure, the sham group did not exhibit evidence of tinnitus [no significant increase in ratio ($p>0.05$) <0.8, (A)]. Prepulse inhibition data showed no behavioral evidence of auditory detection deficits 35 days after blast exposure: [(1) 35 days after the blast exposure, the blast group did not exhibit significant increase in PPI ratio at any frequency bands ($p<0.05$) and (B); (2) 35 days after the blast exposure, the sham group exhibited significant decrease in PPI ratio at 20 kHz ($p<0.05$) (B)]. Error bars represent SEM.

When all the blasted rats were grouped together (n=13), the average gap detection and PPI data were compared against those of the sham group. As seen in Figure 12, the blasted group exhibited strong behavioral evidence of tinnitus at 26~28 kHz (Figure 12A), and no significant change in PPI ratios ($p = 0.63$) (Figure 12B).

*Elevated plus maze testing for anxiety*

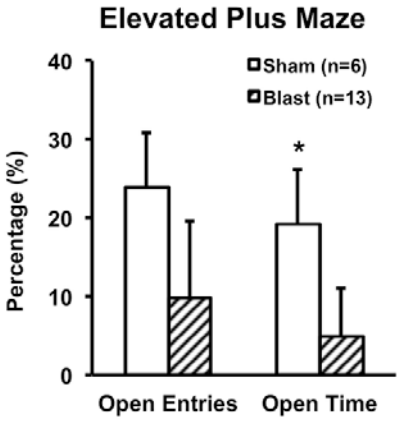



Figure 13. Percent of open-arm entries and open-arm time in the elevated plus maze. Compared to the sham group, blasted rats made significantly less entries to the open-arms and spent significantly more time on the open-arms ($p<0.05$), indicating significantly higher anxiety levels. Error bars represent SEM.

Rats in the sham group committed an average of 32.6±7.7% of open-arm entries and spent an average time of 26.2±6.8% percent on the open arms. Rats in the blasted group committed an average of 9.8±4.7% of open-arm entries and spent an average time of 4.8±2.8% in the open arms. Compared to blasted rats, rats in sham group spent significantly more time in the open arms ($p<0.05$) and made significantly more entries into the open arms ($p<0.05$) (Figure 13). This clearly demonstrated that blast exposure induced significant anxiety.

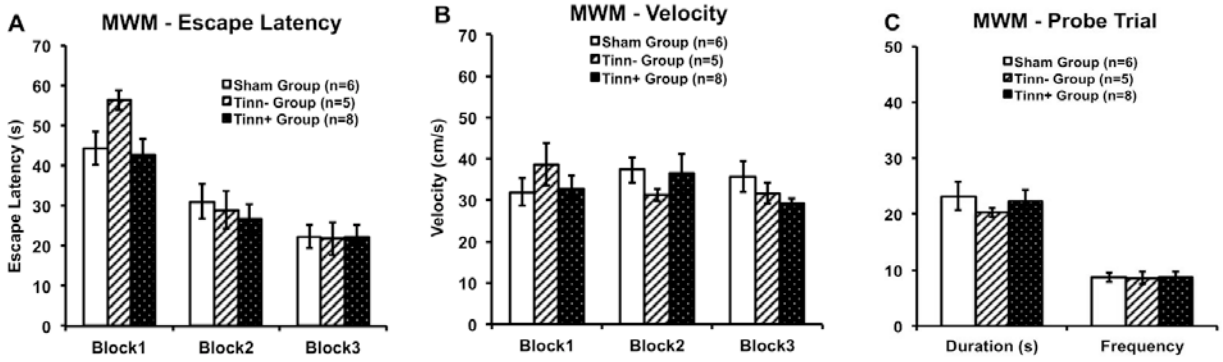



Figure 14. Morris water maze escape latency, velocity, and probe trial data. No significance differences were seen among the tinnitus (+), tinnitus (-), and sham group in escape latency (A), velocity (B), or probe trial target zone entries or time (C) ($p>0.05$), indicating similar spatial learning and memory across groups. Error bars represent SEM.

*Morris water maze test for cognitive deficits*

*Spatial task acquisition*. Three blocks comprised of four trials apiece served as the spatial acquisition task trials (Figure 14A & B). There was no significant difference in the latency and velocity among the three groups in blocks 1 through 3 ($p>0.05$), indicating there was no difference in the spatial learning among the three groups.

*Probe trial acquisition*. No significant differences in target zone entries or time were observed between groups in the probe trial ($p>0.05$, Figure 14C). These results mirror our recent findings that not all tinnitus positive animals develop cognitive impairment (Pace and Zhang, 2013).

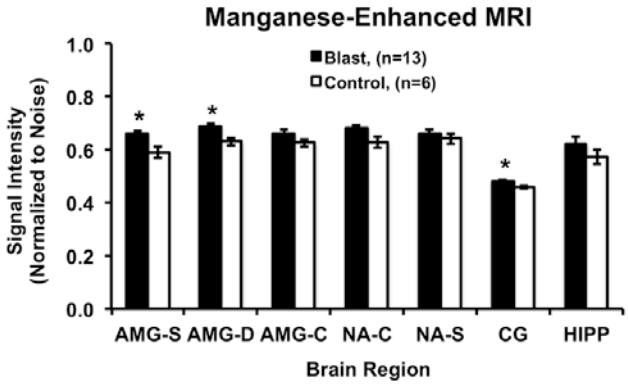



Figure 15. The average bilateral signal-to-noise ratios of limbic and paralimbic structures. Compared to the sham group, the blast group showed higher Mn2+ uptake in the superficial

(AMG-S) and deep (AMG-D) subdivisions of the amygdaloidal complex and anterior cingulate cortex ($p<0.05$).

When the tinnitus (+) and tinnitus (-) groups were combined into the blast group (n=13), the resulting group averages of SNRs were compared with those of the sham group. We found that, compared to the sham group, the blast group demonstrated higher manganese uptake in bilateral superficial and deep groups of the amygdaloidal complex and anterior cingulate cortex ($p < 0.05$), as seen in Figure 15.

**6. Traumatic brain injury (TBI) following induction of tinnitus with blast exposure.**

Following investigation of neural activity changes through electrophysiological recordings and MEMRI, we examined TBI by assessing glial reactivity and axonal degenerative changes in the dorsal cochlear nucleus (DCN), inferior colliculus (IC) and auditory cortex (AC) following exposure to blast overpressure. Both glial reactivity and axonal degenerative changes were assessed by performed glial fibrillary acidic protein (GFAP) immunohistochemistry and Silver staining for degenerating axons.

*GFAP immunohistochemistry*

For quantitative analysis of reactive astrocytosis in DCN, IC and AC regions, 5 representative sections per animal from each region were subjected to antigen retrieval by incubation in a citrate buffer (pH6.0) at 90$^{o}$C for 1 hour followed by immersion in 0.3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were then incubated overnight in a mouse anti GFAP antibody (NE1015, EMD chemicals, Gibbstown, NJ) diluted in 2% normal goat serum (Vector Laboratories, Burlingame, CA) in 1% bovine serum albumin (BSA). Sham sections were also incubated in anti GFAP antibody. The sections were then incubated in biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA) followed by exposure to Vectastain Elite ABC reagent and chromogen development by diaminobenzidine. In control incubations, normal goat serum was substituted for primary antibody. All sections were observed under a light microscope (Leica DMLB, Leica Microsystems Ltd, Heerburg, Switzerland) to visualize GFAP reactive astrocytes.

*Quantitative analysis of reactive astrocytosis*

To quantify the extent of astrocytosis, 10 representative digital images (x20 magnification) from each section encompassing bilateral regions of the DCN, IC or AC were obtained. Then the total number of identifiable astrocytes in each digital image was counted by a blinded observer using the cell counter function in ImageJ (http://rsb.info.nih.gov/ij/). The average number of astrocytes per group and region were calculated and statistically compared for group-wise differences using one-way analysis of variance or t-test using SPSS (IBM).

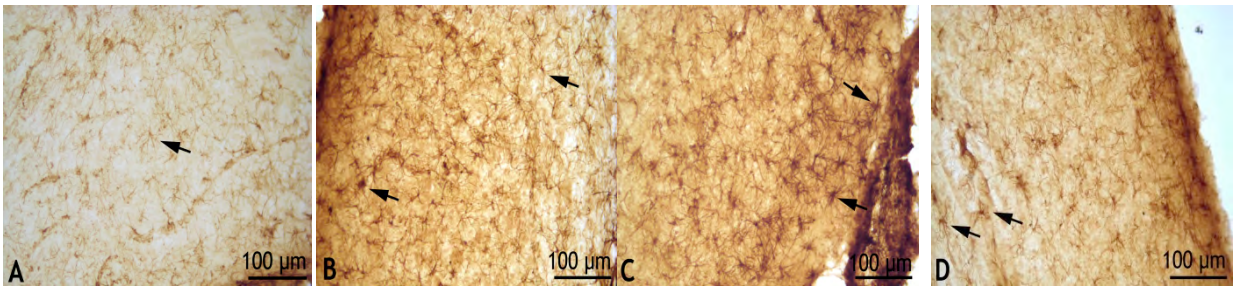



Fig. 16. Astrocytic reaction in brain section encompassing DCN. Figure1 6A shows GFAP reactive astrocytes in sham sections. Figure 6B shows astrocytic reaction 1day after blast overpressure exposure. Figure 16C shows astrocytes in DCN 1 month after exposure. Figure 16D shows GFAP reactivity in region of AC after 3 months.

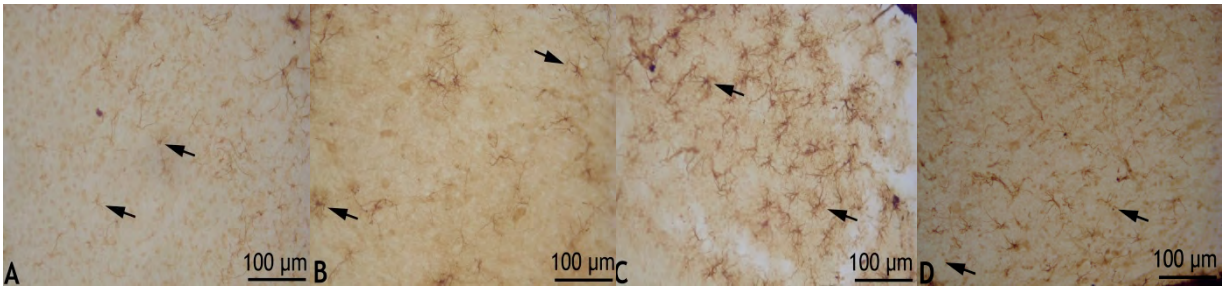



Figure 17. Astrocytic reaction in brain section encompassing IC. Figure 17A shows GFAP reactive astrocytes in sham sections. Figure 17B shows astrocytic reaction 1 day after blast overpressure exposure. Figure 7C shows astrocytes in IC 1 month after exposure. Figure 17D shows GFAP reactivity in region of AC after 3 months.

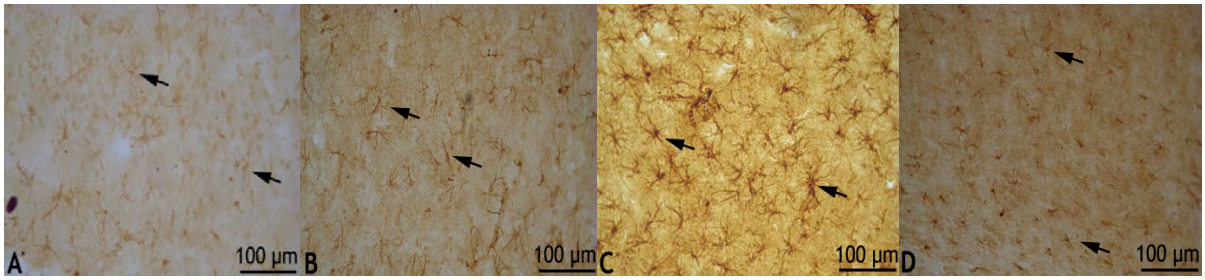



Fig. 18. Astrocytic reaction in brain section encompassing AC. Figure 18A shows GFAP reactive astrocytes in sham sections. Figure 18B shows astrocytic reaction 1day after blast overpressure exposure. Figure 18C shows astrocytes in AC 1 month after exposure. Figure 18D shows GFAP reactivity in the region of AC after 3 months.

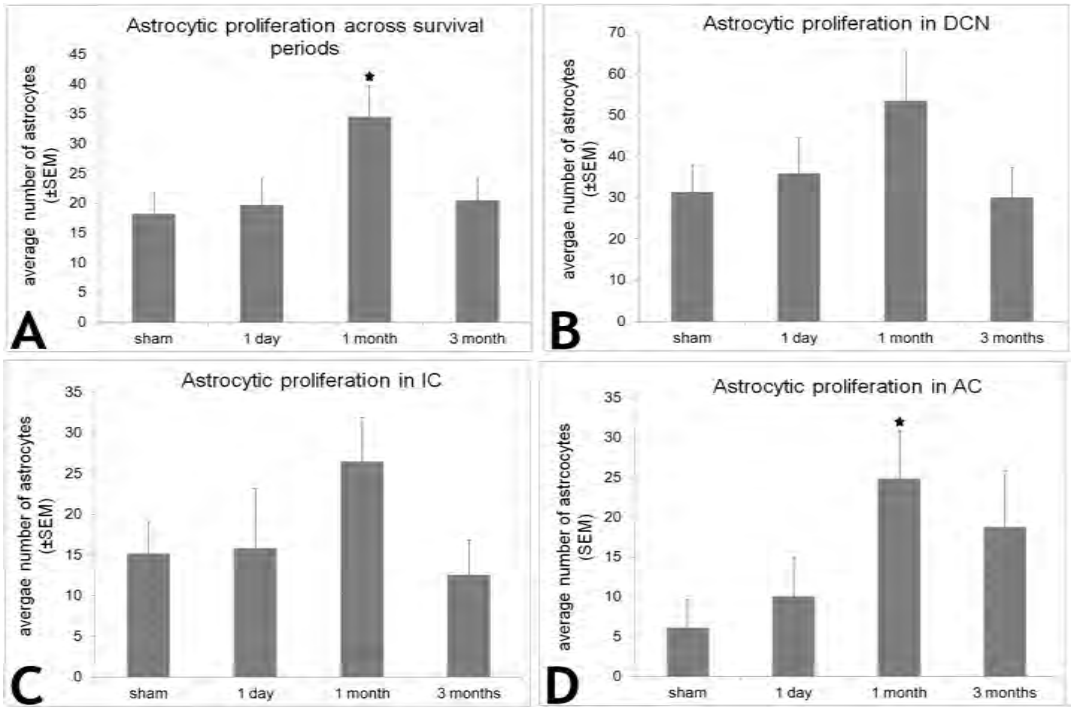


Figure 19. Quantified data. Panel A shows the extent of astrocytic proliferation at 1 day, 1 month and 3 months after overpressure exposure. Astrocytic proliferation was significantly high (*p<0.05)* 1 month after overpressure exposure compared to other groups. Panel B shows the extent of astrocytic proliferation in DCN sections In DCN, although considerably elevated astrocytic proliferation was observed at 1 day and 1 month post blast. Panel C shows astrocytosis in sections from IC. Astrocytosis was considerably high at 1 month after blast exposure. Panel D shows astrocytosis in sections from AC. In AC, the extent of astrocytic proliferation was significantly high at 1 month period after exposure that stayed elevated even 3months after blast.

Observation of sections from sham, 1 day, 1 month and 3 months post blast revealed the presence of astrocytes in all the brain regions. However, quantification on the extent astrocytosis was limited to representative regions in the DCN (Figure 16), IC (Figure 17) and AC (Figure 18), considering their predominant involvement in auditory functions.

Quantification of the extent of reactive astrocytic proliferation revealed significantly higher astrocytosis in blast exposure sections harvested after 1 month compared to shams (*p <0.05;* Figure 19A*)*. A further analysis of astrocyte count in the DCN alone showed an increase in the number of astrocytes by 1 day after blast that stayed elevated up to 1 month after blast (Figure 19B). In the IC, the most pronounced increase in the number of astrocytes was observed in sections studied at 1 month after blast (Figure 19B). Analysis of representative AC regions revealed a temporal elevation in the number of astrocytes (Figure 19C). There was a marked increase in the number of astrocytes by 1 day with a significant increase by 1 month (*p=<0.05)* compared to sham. At 3 months after blast exposure, the number of astrocytes was still elevated compared to sham.

*Silver staining for degenerating axons*

For qualitative analysis of the extent of blast overpressure induced axonal injury in sections encompassing the DCN, IC and AC, a separate set of 5 representative sections from each region per rat were subjected to a previously described silver impregnation technique (Gallyas et al., 1980) that was also used to demonstrate diffuse axonal injury (Kallakuri et al., 2003, Kallakuri et al., 2008). The sections were immersed for 3 min in pretreatment solution (equal volumes of 9% sodium hydroxide and 15% hydroxylamine) followed by a wash in 0.5% acetic acid (3x3 min) or until the sections turned opaque. The sections were then incubated in an impregnation solution (5 mg/ml ferric nitrate and 100 mg/ml silver nitrate) for 30 min. They were then washed in 1% citric acid (4x2 min) followed by a wash in 0.5% acetic acid for 5 min. Then they were placed in a developer solution until they turned pale gray. After sufficient development, they were removed and washed thoroughly in 0.5% acetic acid (3x10 min), rinsed in distilled water, mounted on a slide and cover-slipped and examined under a light microscope (Leica DMLB, Leica Microsystems Ltd, Heerburg, Switzerland) for degenerating axons.

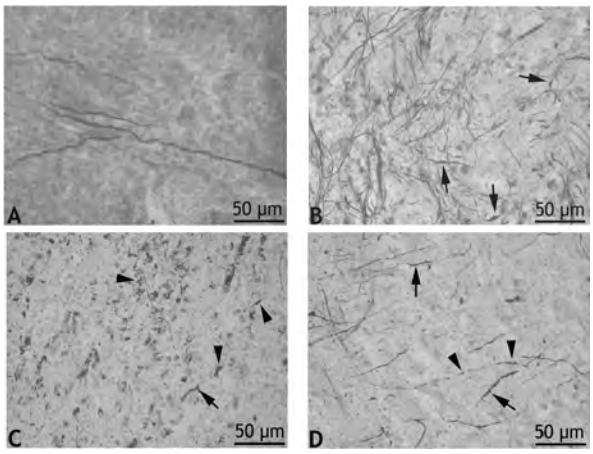



Figure 20. The extent of degenerating axons in various auditory centers. Figure 20A shows normal appearing axons in sham sections. Axons with uniform caliber could be seen coursing through a region encompassing DCN. Figure 20B shows degenerative changes in DCN. In this image from a 3 month post blast section, injury changes in the form of axonal swellings (arrows) in a region between DCN and ganglion of the spinal nerve 5 could be seen. Figure 20C shows degenerating changes in IC 1 month after exposure. Axonal swellings (arrow), retraction balls and debris (arrow heads ) could be seen. Figure 20D shows axonal changes in AC. Swollen axons (arrows) and axons with vacuoles and retraction balls (arrow heads) could be observed in

thalamic region besides others.

As shown in Figure 20, silver stained representative sections encompassing the DCN, IC and AC from sham and blast exposure groups were investigated for the presence of degenerating axons. Observation of sham sections revealed long tracts of axons with uniform caliber in sections encompassing the DCN, IC and AC. In all the sham sections, axons could be seen coursing through long distances uninterrupted in various white matter tracts (Figure 20A). In the case of blast exposed sections, axons at various stages of degeneration could be found in many regions: trapezoid, spinal trigeminal tract, pyramids, ventral spinocerebellar tract, medial longitudinal fasciculus, transverse fibers of pons, pons, tracts of lateral lemniscus, commissure of inferior colliculus and occasionally in the most posterior aspects of the corpus callosum. In sections encompassing the DCN, degenerating axons could be found at 1 day, 1 month and even at 3 months after exposure in various tracts such as the trapezoid, spinal trigeminal tract, pyramids, ventral spinocerebellar tract and medial longitudinal fasciculus (Figure 20B). In sections from 1 day after exposure, axons could be found with signs of increased inter-axonal spacing and swellings could be found in tracts such as spinocerebellar tract and axons in the cochlear nucleus. In DCN sections from 1 month, degenerating axons could be found with swellings and beads along with dark stained axons associated with axonal debris. Also found were axons with vacuolations. In DCN sections from 3 months after exposure, the incidence of axonal degeneration appears to be more preponderant as indicated by higher incidence of axonal swellings, axons with uneven caliber, and even retraction balls. Furthermore, the extent of uneven caliber axons appears to be more intense as revealed by a high number of wrinkled axons. By 3 months after exposure, degenerating axons could be found in long tracts such as medial longitudinal fasciculus.

In sections encompassing the IC, the brainstem regions involved were white matter tracts including transverse fibers of the pons, pons, lateral lemniscus and commissure of inferior colliculus (Figure 20C). In IC sections 1 day after the exposure, degenerating axons could be observed in the form of axonal beads, swellings and vacuoles. The vacuolated axons could be found in the trapezoid and other white matter tracts such as the lateral lemniscus. Furthermore, in these sections, large caliber axons were prominently found and appeared as swollen axons. IC sections at 3 months post blast had a preponderance of degenerating axons compared to IC sections from 1 month after blast. In IC sections from 3 months post blast, degenerating axons appeared to be similar to those from 1 day after exposure with the number of wrinkled axons appearing more prominently. In sections encompassing AC, degenerating axons at various stages could be observed in regions of the corpus callosum, fimbria of hippocampus, optic radiations, and areas of thalamus (Figure 20D). These degenerative changes in the form of axonal debris and wrinkled axons were predominantly found in sections at 1 day and at 3 months after blast.

## B. CONCUSSION-INDUCED TINNITUS AND HEARING LOSS

This is the second phase of our project. Rats were subjected to a single traumatic brain injury (TBI) insult using the Marmarou impact acceleration (IA) injury model. No skin incision was made in these rats unlike the traditional Marmarou model that involves exposing the skull through a longitudinal skin incision exposing the periosteum to affix a steel disc (10 mm diameter; 3 mm thickness) at the middle between bregma and lambdoid sutures. Rats were anesthetized (4% Isoflurane +0.6L/min oxygen) for 4 minutes in an induction chamber and then were positioned in a prone position on a foam bed contained in Plexiglas box. Then, 450 g cylindrical brass weight (18mm diameter) was dropped directly onto the scalp. The impact location would correspond approximately to a location between bregma and lambdoid sutures. Sham rats were subjected to anesthesia alone but not injured. Rats were subjected to 1) mild TBI by dropping the impactor from a height of 1.0m, 2) moderate TBI by dropping the impactor

from a height of 1.5 m, and 3) severe TBI by dropping the impactor from a height of 1.8 m and 2.0 m respectively. Immediately after the impact, rats were removed to avoid a second impact and monitored for latency to surface right considered as an indirect indicator of loss of consciousness.

Rats were tested for tinnitus using gap-detection procedure and for auditory detection and hearing using prepulse inhibition and ABR procedures, respectively. Mild TBI (1.0 m height impactor weight drop) was initially investigated, but was not further explored due to lack of sustained anatomical, physiological, and behavioral impairment.

*a) ABR data*. Immediately following concussion (Post-Conc. Day 0-1), there were no significant hearing threshold shifts among the weight-drop groups in either ear.

*b) Behavior results*. Immediately following trauma (Post-Day 0), all concussed groups showed significantly elevated gap detection ratio values across the majority of frequency bands, in comparison to baseline (Pre) gap detection values (Figure 21a-f). PPI ratio values are similarly increased. These data suggest that while concussion exposure may have induced tinnitus, deficits in central auditory detection were also induced and may play a role in the apparent tinnitus behavior. At one week post-concussion, the majority of gap-detection and PPI values had returned to baseline levels, and by two weeks post-concussion, gap-detection and PPI values had completely recovered to baseline levels. Taken together, these data indicate that concussion may induce immediate tinnitus and auditory detection deficits, however this impairment mostly disappears within one week. Additionally, using 2.0 m height for impact acceleration does not seem to cause significantly greater behavioral impairments than using the 1.5 m height. We are currently subjecting rats to repeated impact acceleration to see if longer lasting behavioral impairments can be obtained.

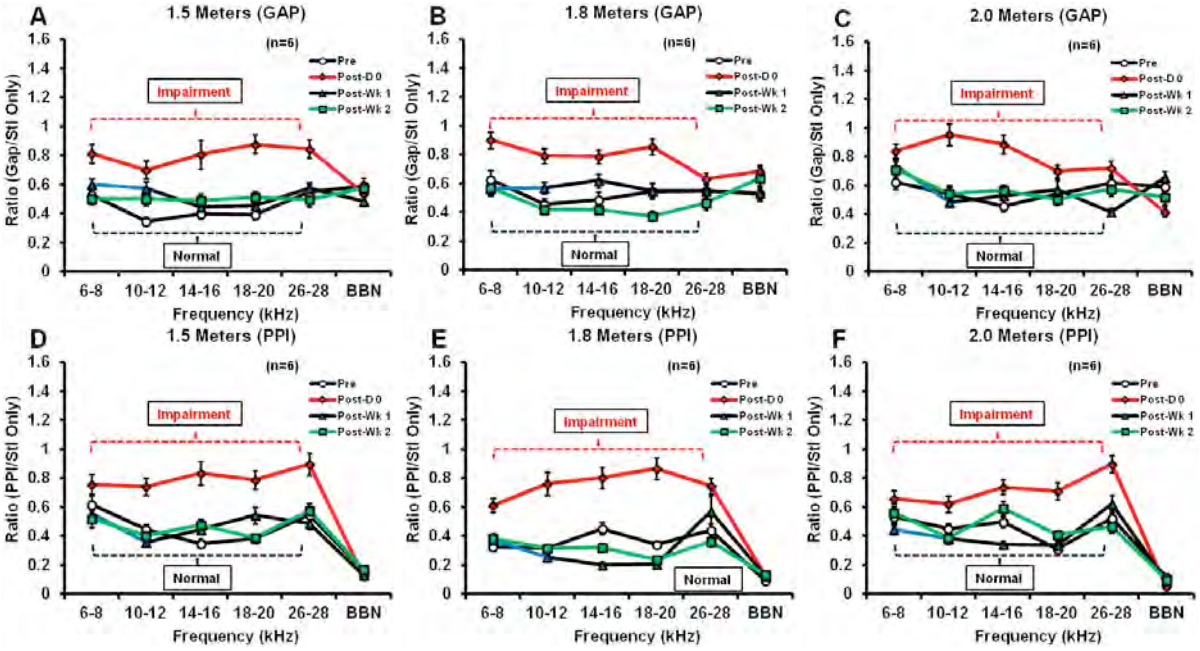



Figure 12. Gap-detection and PPI behavioral data showing tinnitus and auditory detection impairments, respectively, immediately following concussion, which mostly recovers by 1 week and completely recovers by 2 weeks.

*c) Concussion histopathology.* Histopathology has been performed on 2 rats exposed to 1.0 m, 1.5 m, and 1.8 m impactor weight drop height. Rats were sacrificed two weeks after TBI for histological observations of axonal injury.

No skull fractures, respiratory depression or seizures were observed in any of the rats. 40 µm thick sections of brainstem were subjected to beta amyloid precursor protein (βAPP) immunocytochemistry. βAPP reactivity is a reliable marker of axonal injury. Rats subjected to severe TBI (1.8 m) exhibited prolonged duration to surface right compared to rats from other groups (Fig. 13a). Rats subjected to TBI from 1.8 m showed axonal injury in regions of brainstem (Fig. 22b) predominantly in the reticular formation and pyramidal tracts. No apparent axonal injury was evident in rats exposed to the 1.0 m impactor weight drop height (Fig. 22c).

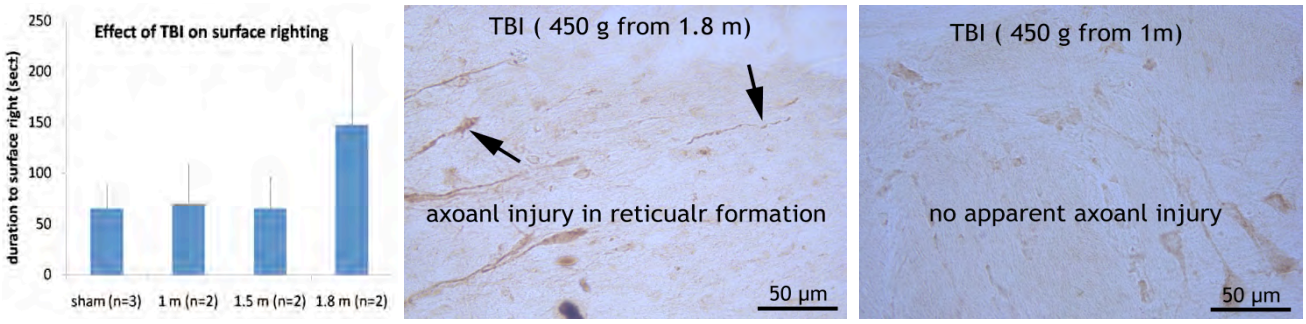


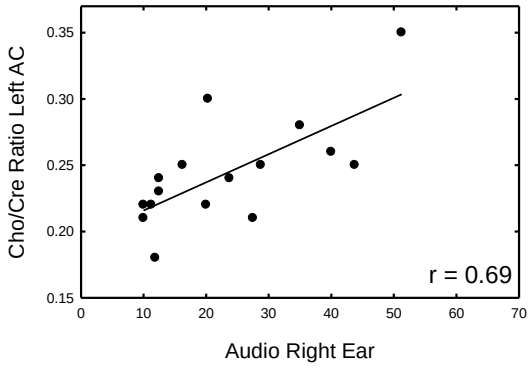

Fig. 22. Image showing surface righting duration in rats subjected to varying severities of TBI (Fig. 22a). Rats subjected to TBI from 1.8 m (Fig. 22b) showed prominent axonal injury while those exposed to 1.0 m (Fig. 22c) did not.

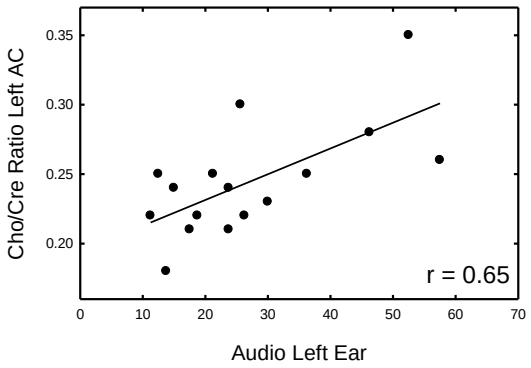
## C. COMBINED BLAST- AND CONCUSSION-INDUCED TINNITUS AND HEARING LOSS

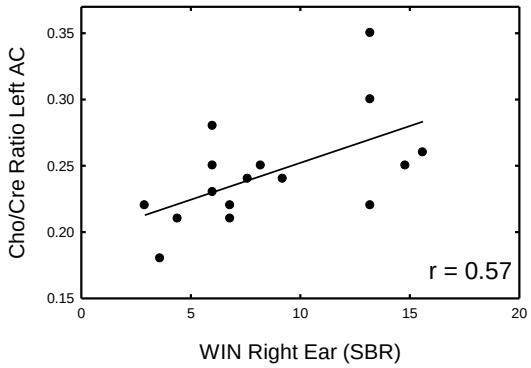
We have initiated this experiment on the first batch of animals towards the end of year 2013. Behavioral and ABR testing was conducted on these rats before and after combined blast and concussion. The level of blast was 22 psi and the height of drop weight to induced concussion was 1.5 m. Post-impact is being evaluated. We will report our progress within the next three months.

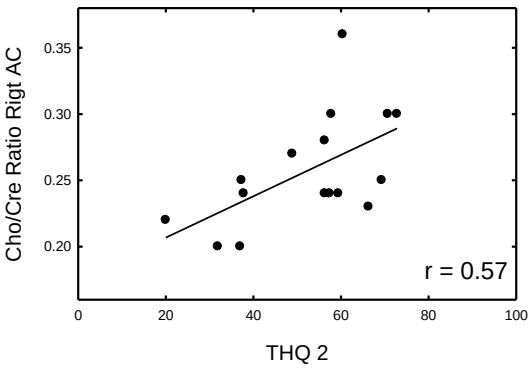
## REVIEW OF HUMAN STUDIES:

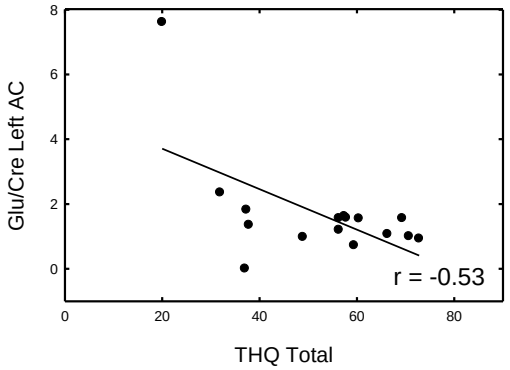
Our current analysis focuses on establishing trends in various imaging procedures in humans (diffusion-tensor imaging, DTI; susceptibility-weighted imaging, SWI; and MR-spectroscopy, MRS), and their relationship to audiometric (hearing loss), psychophysical (word recognition in quiet and noise, loudness), psychometric (response to questionnaires), and neuropsychological data in those participants with blast induced hearing loss and tinnitus. In the last quarterly report, we described trends in the MRS, audiometric, and neuropsychological assessments. We now have increased the sample size and included additional variables. Examples of the MRS related auditory data are present in the composite graphs below.

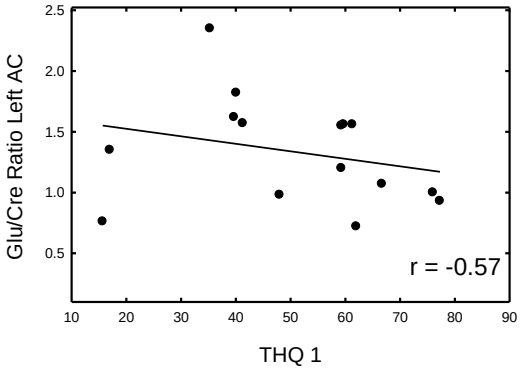


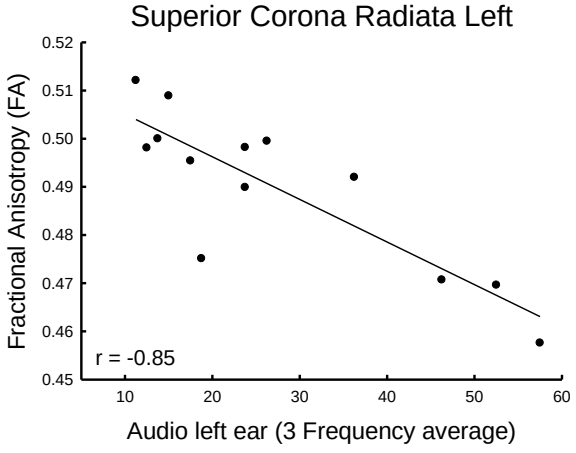



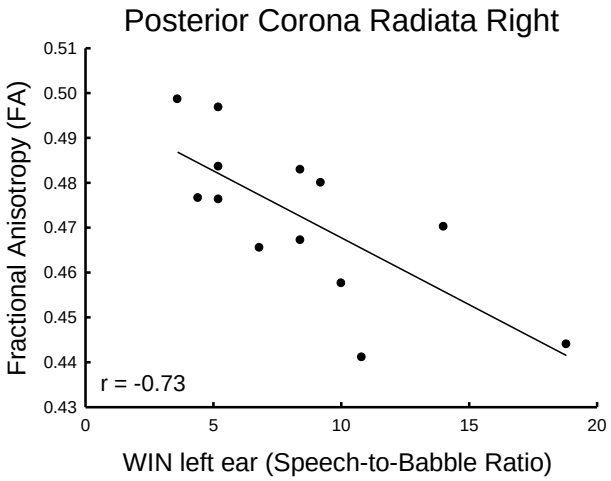
In participants with blast induced hearing loss and tinnitus, data show that increased hearing loss in the left and right ear is associated with increased choline levels in the left auditory cortex (AC), poorer performance (increase speech-to-babble ratio, SBR) on the Words-in Noise (WIN) test, poorer performance (perceived effect) on Tinnitus and Hearing Loss based subscale on the Tinnitus Handicap Questionnaire (THQ1). With respect to the excitatory neurochemical glutamate (Glu), we found inverse relations between Glu and the total THQ score and THQ subscale (1). In other words, there was a reduction in GLU) which was related to perceived increases (poorer scores) on social, behavioral, and emotional characteristics of the THQ subscale.

Diffusion-tensor imaging (DTI) was also evaluated in individual participants across relevant white matter sites in the left and right hemispheres of the brain including: genu, splenium, and body of the corpus callosum, fornix, corticospinal tracks, medial thalamus, inferior and superior cerebellar peduncle, anterior, superior, posterior corona radiata, superior and inferior longitudinal fasciculus, etc.). While DTI represents a relatively new neuroimaging modality, it is particularly important because it can provide insight into plastic/reactive changes in white matter microstructure and connectivity associated with tinnitus that cannot be detected by conventional MRI. Specifically, DTI measures the displacement of water molecules (diffusion) within white matter tracts, providing information on the microstructure of cerebral white matter and thus serves as a biomarker of tissue integrity. Consequently, for each voxel, DTI estimates diffusion in three orthogonal axes (eigenvectors) of an ellipsoid, defining the principal (major), intermediate, and minor axes. The most commonly used metric to quantify the relationship between eigenvalues is fractional anisotropy (FA), a normalized scalar that represents the fraction of the diffusion tensor which is anisotropic. In the analyses and graphs presented herein, we focus on FA because it reveals information regarding fiber integrity and network organization/reorganization, i.e., activity dependent neuroplasticity. The FA metric ranges between 0 and 1, where 0 represents perfectly "isotropic" diffusion, such as is found in the cerebrospinal fluid where diffusion is equivalent in all directions, and where 1 is the extrema for "anisotropic" diffusion, indicating maximum difference between directional components, such as is found in coherent white matter tracts which consist of long tubes.

**DTI: Audiometric, psychoacoustic, and questionnaire data**

With respect to the audiometric data (average audiometric thresholds for the left and right ear, word-in-noise (WIN) test for left and right ear, monosyllabic word recognition testing for the left and right ears, loudness level estimates, and Tinnitus Handicap Questionnaire performance for the two subscales and total scores) and the main DTI metric (FA), we document significant inverse relationships with audiometric thresholds of the left ear with the superior and posterior corona radiate on the left and right side of the brain (SCR-R, r = -.72; SCR-L, r = -.85; PCR-R, r = -.70; PCR-L, r = -.56); and the external capsule, EC-R (r = -.84; EC-L, r = -.60). Two graphic representations of these relationships are shown below.

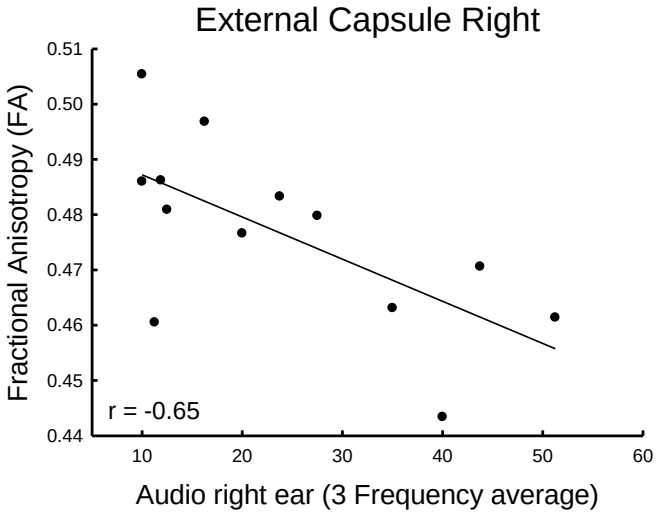





Audiometric thresholds for the right ear showed similar findings (SCR-R, r = -.63; SCR-L, r = -67, PCR-R, r = -.64; EC-R, r = -.65).

Words-in-noise test for the left ear demonstrated relations with the retrolenticular portion of the internal capsule, RLIC-R, r = -.69; PCR-R, r = -.73, PCR-L, r = -.60; and external capsule EC-R, r = -.59).

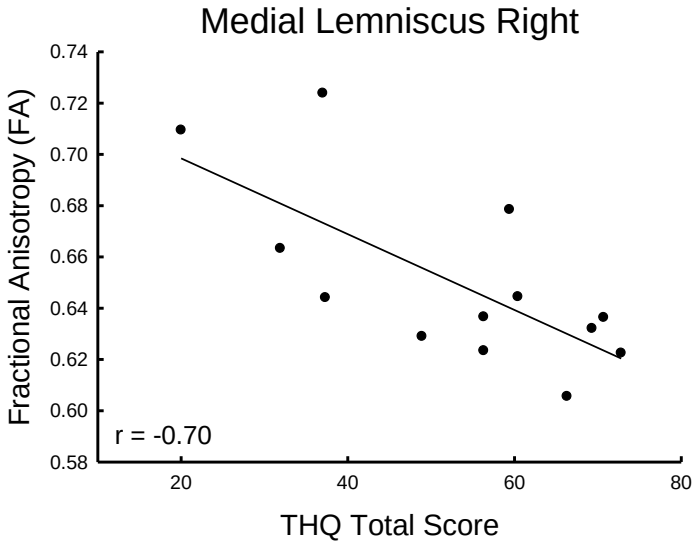
WIN test for the right ear only demonstrated a relation with the EC-R, r = -.68.

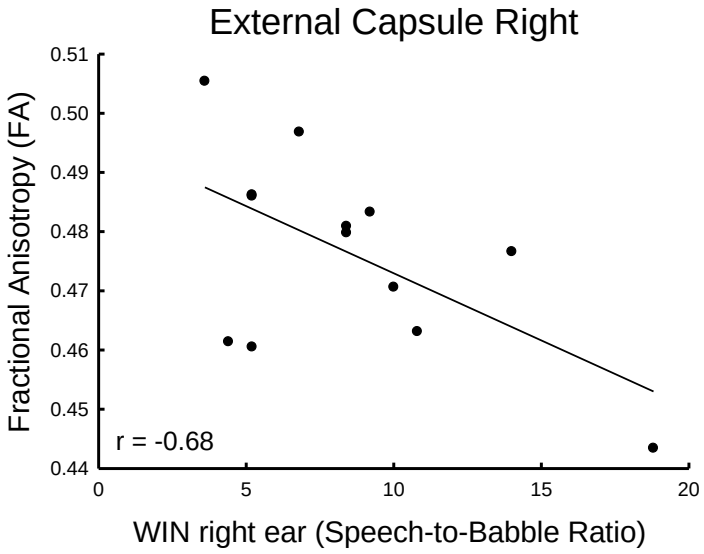



Monosyllabic word recognition in quiet for the left ear only demonstrated a relation with the SCR-L, r = .63 (i.e., the only positive correlation); right ear word recognition performance for the right ear failed to show any significant relations.

Loudness level measures showed a relationship with the fornix, r = -.73; anterior thalamic radiation on the right and left sides (ACR-R, r = -.64; ATR-L, -.67) and CR-R, r = -.69; CR-L r = -.60.

The THQ data showed relationship with the medial lemniscus (ML) on the right side, THQ1, ML-R, r = -.71, THQ2, ML-R, r = -.60; THQT, ML-R, -.70).



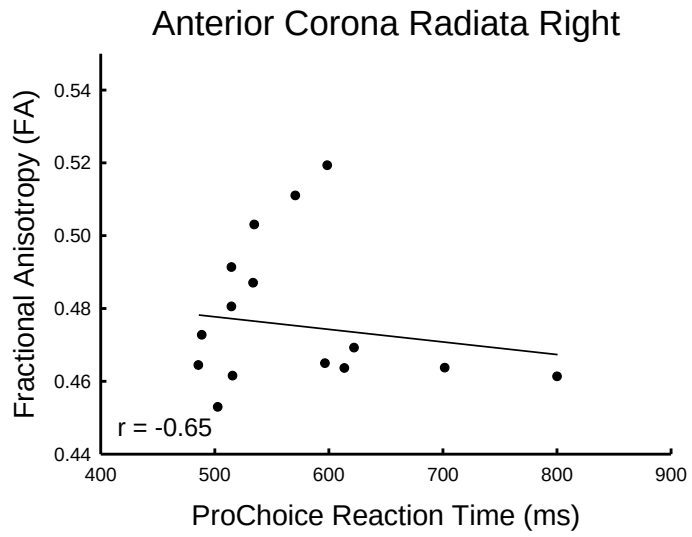



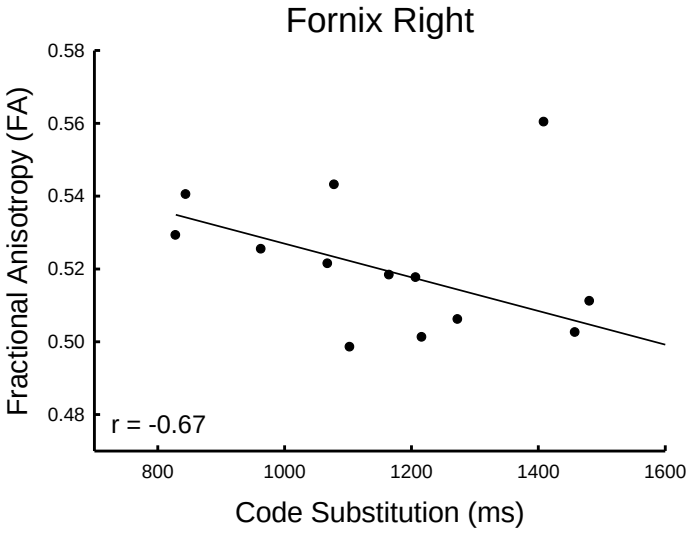

**DTI: Selected Neuropsychological Variables**

Simple reaction time failed to show any distinct relationships with the DTI metric.

Procedural choice reaction time demonstrated significant inverse relationships with the fornix, r = -.54, anterior corona radiata on the right and left side, r = -.64, -.61; sagittal striatum SS left, r = -.59; cingulate gyrus-L, r = -.5; fornix-R, -.61. Math processing showed a significant relation with the body of the corpus callosum, r = -.58. Other relationships such as matching-to-sample, showed relationships with the superior cerebellar peduncle (SCP) right and left, r = .67, .63; anterior limb of the internal capsule (LIC) left and right, r = .63, .64; uncinate fasciculus-R, r = .52. Code substitution showed relations with the SCP-Right, r = .56, SCP, left, r = .56; ACR right and left, r = -.59. -.56; SS left, r = -.57; FOR right and left, -.67, -.59.

Code substitution delayed showed a positive relation to RLIC R, r = .53.




We also present new data on susceptibility-weighted imaging (SWI) with metrics that include: 1) average iron of the total region, 2) total iron of high content regions, and average iron of the high content regions. We focus on the following brain left and right brain regions: caudate nucleus, globus palladus, pulvinar, putamen, red nucleus, substantia nigra, and thalamus. Consideration is given to the fact that the source of iron deposition may be myelin/oligodendrocyte debris, concentrated iron in the macrophages (i.e., that phagocytize the destructed myelin/oligodendrocyte), the product of hemorrhages from damaged brain vessels, or other factors. There is also reason to believe that the mechanism of direct damage to the brain by iron might also be related to oxidative stress and the generation of toxic free radicals. Moreover, the amount of iron deposition could reflect the extent of tissue damage, thus iron could be used as a biomarker to predict the relationship with auditory psychophysical and psychometric outcome variables.

**SWI Audiometric Data: Average iron**

Audiometric thresholds for the left ear showed increased iron in Putamen-L, r = .54; right side was non-significant. WIN test for the left ear showed a relation with Pulvinar-R, = r.52; Pulvinar-L, r = .62, Thalamus-R, r = .58. Word Recognition in quiet for the left ear show a positive relation to the Putamen-R, r = .52 and Putamen-L, r = .63. No other psychoacoustic measures showed any significant relationships.

**SWI Audiometric Data: Total iron**
AUD- L no relations; AUD-R, Putamen-R, -.53. WIN-L Pulvinar-R, r = .59, Pulvinar-L, r = .59.

**SWI Audiometric Data: Average iron region II.**
AUD-R, Putamen-R, -.53.WIN-L Pulvinar-R, r = .59; Pulvinar-L, r = .59.

Cognitive measures from ANAM used in this analysis include: 1) Simple reaction time (SRT), repeated twice to evaluate for fatigability; 2) Procedural (Choice) RT (PRO); 3) Mathematical Processing (MTH); 4) Matching-to-Sample (Working Memory; MTS); 5) Code Substitution (Processing Speed; CDS); and 6) Code Substitution Delayed (delayed incidental recall; CDD).

**SWI Neuropsychological Data: Average iron**
Procedural choice reaction time showed an inverse relation with Caudate-L, r = -.52

**SWI Neuropsychological Data: Total iron**
Code substitution (processing speed), Thalamus-R, r = -.5.

**SWI Neuropsychological Data: Average iron region II**
No significant relationships were found.

With respect to auditory and neuropsychological data, we find the following relationships. Audiometric sensitivity in both ears was consistently related to mean reaction time for correct responses during processing speed (CDS) and delayed incidental recall (CDD) tasks, with correlations ranging from .61 to .73 (p's < .03). Both of these processing speed measures were also modestly related to performance on the Words in Noise (WIN) task for the right ear only (CDD, r= .53, p < .05, CDS, r = .44, p = .10). Significant negative relationships between processing speed and the WREC task for the left ear were also observed. Intra-individual variability in RT on CDS (processing speed) and CDD (delayed incidental recall were also associated with audiometric sensitivity in each ear (r's = .49 to .70, p's < .06 to < .01) for both ears. A marginally significant relationship was observed between performance accuracy during delayed incidental recall and audiometric sensitivity in the left and right ears (r's = -.41 and -.45, p's = .13 and .09, respectively). Throughput (number of correct responses per minute) during processing speed and delayed incidental recall also showed significant to marginally significant relationships with audiometric sensitivity in the left and right ears (p's ranging between .03 and .07).

## KEY ACCOMPLISHMENTS

**Animal Studies:**

- Although blast exposure induces temporary hearing threshold shift, there is persistent/chronic degradation in the P1-N1 amplitude of auditory brainstem responses. This indicates that blast exposure permanently compromises hearing and that the currently used rat model is appropriate to study blast-induced hearing impairment and tinnitus.
- Blast trauma induces onset and chronic tinnitus. The induced chronic tinnitus shifts towards the high-frequency region over time.
- When studying neural activity changes along the auditory axis, we found that blast

trauma induces onset hyperactivity and increased bursting activity in the auditory brainstem, which later shifts to the auditory cortex.

- The induced tinnitus by blast impact is accompanied by increased neuronal activity in limbic structures such as the amygdala and anterior cinculate cortex and by increased anxiety. This demonstrates the involvement of both auditory and limbic structures in the etiology of blast-induced tinnitus.
- Blast exposures induces traumatic brain injury (TBI) as revealed by sustained astrogliosis and axonal injury in auditory brain centers.
- The continued changes in the blast trauma-induced tinnitus, hearing impairment, hyperactivity in both auditory and limbic structures, and TBI demonstrate a continued maladaptive plasticity in both auditory and non-auditory structures, which underlie blast-induced tinnitus.
- Concussion may induce onset rather than chronic tinnitus and auditory detection deficits. In general, the induced effects are accompanied by moderate level of axonal injury.

**Human Studies:**

- In individuals with blast induced hearing loss and tinnitus, we found that there are significant relations among hearing, psychometric, neuropsychological variables and neuroimaging related parameters; most notably metabolites and neurotransmitters and white matter tracks vis-à-vis the FA metric.
- Blast induced hearing loss and tinnitus is not clearly associated with MRI susceptibility weighted imaging (SWI) variables.
- Several aspects of processing speed and delayed incidental recall during computer-based cognitive evaluation are associated with tinnitus severity.
- Specifically, variations in audiometric sensitivity appear to be related to performance accuracy, reaction time, and intra-individual variability on measures of processing speed and delayed-incidental recall.
- Individuals with blast-induced hearing loss and tinnitus are consistent with previous research on patients with chronic tinnitus which imply that slowed reaction time, increased reaction time variability, and lower accuracy may be associated with difficulty inhibiting attention to distracting tinnitus signals.

**REPORTABLE OUTCOMES:**

**Animal Studies:**

***Articles:***

Anthony T. Cacace, Tom Brozoski, Bruce Berkowitz, Carol Bauer, Boris Odintsov, Magnus Bergkvist, James Castracane, JinSheng Zhang, Avril Genene Holt “Manganese enhanced magnetic resonance imaging (MEMRI): 2 A powerful new imaging method to study tinnitus”, *Hearing Research.*

Jessica Ouyang, Edward Pace, Laura Lepczyk, Michael Kaufman, Wei Zhang, and Jinsheng Zhang (being revised) Limbic involvement in blast-induced tinnitus, anxiety and related post-traumatic stress disorder in rats. *Experimental Neurology.*

Gulrez Mahmood, Zhigang Mei, Houmehr Hojjat, Edward Pace, Jinsheng Zhang (being reviesed) “Therapeutic Effect of Sildenafil on Blast-Induced Tinnitus and Auditory Impairment”, *Neuroscience*.

Hao Luo, Xueguo Zhang, Edward Pace and Jinsheng Zhang (being revised) Blast-Induced Tinnitus and Spontaneous Firing Changes in the Rat Dorsal Cochlear Nucleus, J. Neurosci Res.

Hao Luo, Xueguo Zhang, Edward Pace and Jinsheng Zhang (To be submitted) Blast-Induced Tinnitus and Spontaneous Firing Changes in the Rat Inferior colliculus, Neuroscience.

Hao Luo, Xueguo Zhang, Edward Pace and Jinsheng Zhang (In completion) Blast-Induced Tinnitus and Spontaneous Firing Changes in the Rat Auditory Cortex, Neuroscience.

Kallakuri Srinivas, Huichao Lu, Luo Hao, Edward Pace, Xueguo Zhang, Cavanaugh John M and Zhang Jinsheng (In completion). Blast overpressure induces long lasting astroglial up-regulation and diffuse axonal injury.

Edward Pace, Laura Lepczyk, Jessica Ouyang and Jinsheng Zhang (in preparation) Blast-Induced Tinnitus and Its Related Traumatic Brain Injury in auditory Centers: A Combined Behavior and Manganese-Enhanced MRI Study

***Abstracts (Podium and poster):***

Luo, L., Zhang, X, Kallakuri, S., and Zhang, J.S. (2013) Blast-Induced Tinnitus and Changesin Spontaneous Firing Rates along the Auditory Axis in Rats, Assoc. Res. Otolaryngol. (ARO International Conference).

**Ross Mayerhoff[(a)], *Gregory Kruper[(a)], Gulrez Mahmood[(a)] and Jinsheng Zhang[(a)(b)] "*The Effect of an Enhanced Acoustic Environment on Noise-Induced Tinnitus in Rats", ARO International Conference

Jinsheng Zhang[1,2], Hao Luo[1], Jessica Ouyang[1], Laura Lepczyk[1], Edward Pace[1], Xueguo Zhang[1], Gulrez Mahmood[1], Srinivasu Kallakuri[3], Huichao Lu[3], Wei Zhang[1], Michael S. Zheng[1] and John M. Cavanaugh[3] "BLAST-INDUCED TINNITUS AND ITS RELATED TRAUMATIC BRAIN INJURY", Tinnitus Research Initiative (TRI) International Conference.

"In vivo identification of hyperactive neural activity in rats with behavioral evidence of tinnitus using MEMRI," Avril Genene Holt, Wayne State University, ARO International Conference

Zhang, J.S. (2013) "*Blast-Induced Tinnitus and Its Related Traumatic Brain Injury"*, MRI Center Summer Class Program, WSU, 06/17/2013.

Zhang, J.S. (2013) "*Multidisciplinary Efforts Towards 'Center for Excellence in Tinnitus Research'"*, 4th Annual Traumatic Brain Injury Workshop, WSU, 11/20/2013.

Edward Pace (Zhang lab, WSU): Noise-Induced Tinnitus Using Individualized Gap Detection Analysis and Its Relationship with Hyperacusis, Anxiety, and Spatial Cognition. P30 conference at the Univ of Michigan

Gulrez Mahmood (Zhang lab, WSU): Therapeutic effect of Sildenafil on Acute Blast Induced Tinnitus and Hearing Loss. P30 conference at the Univ of Michigan

**Human Studies**
***Articles:***

Benson RR, Gattu R, Cacace AT. Left hemisphere fractional anisotropy increase in noise-induced tinnitus: A diffusion tensor imaging (DTI) study of white matter tracts in the brain. Hear Res (E-pub ahead of print)

Mahoney MJ, McFarland DJ, Carpenter MS, Rizvi S, Cacace AT. Reliability of broadband middle-ear power-reflectance in younger and older adults: Application of Generalizability Theory. Am J Audiol. (E-pub ahead of print)

Cacace AT, McFarland DJ. (2013). Factors influencing tests of auditory processing: A perspective on current issues and relevant concerns. J Am Acad Audiol. 24, 572-589

***Abstracts (Podium and poster):***

"New approaches for studying tinnitus: bridging the gap between basic science and current clinical concerns," Anthony T. Cacace, Wayne State University, ARO International Conference.

Woodard JL, May PE, Sugarman MA, Norman AL, Cacace, AT. A neuropsychological profile of blast-induced tinnitus. Paper to be presented at the Annual Meeting of the International Neuropsychological Society, February, 2014, Seattle, WA

"Biofabricated manganese encapsulated nanoparticles: Novel theranostic applications to identify and treat tinnitus," James Castracane, College of Nanoscale Science and Engineering, University at Albany, ARO International Conference

INVESTIGATING THE BRAIN "CONNECTOME" OF BLAST-INDUCED TINNITUS: SOME INITIAL OBSERVATIONS OF A RESTING-STATE FUNCTIONAL MAGNETIC RESONANCE IMAGING (RS-*f*MRI) CONNECTIVITY ANALYSIS, AT Cacace, Y Ye, Tinnitus Research Initiative (TRI) International Conference.

A NEUROPSYCHOLOGICAL PROFILE OF BLAST-INDUCED TINNITUS, JL Woodard, PE May, MA Sugarman, AL Norman, AT Cacace, Departments of Psychology and Communication Sciences & Disorders, Wayne State University, Detroit, Michigan, Tinnitus Research Initiative (TRI) International Conference.

Anthony Cacace (WSU): Voxel-Based Morphometry Analysis of Vestibular Related Traumatic-Brain Injury: Preliminary Results. P30 conference at the Univ of Michigan

## CONCLUSION:

Animal studies:

Blast causes onset tinnitus, which latter shifts to high-frequency tinnitus. Blast induces onset hyperactivity in the lower auditory brainstem, which later shifts to the auditory cortex. In the MEMRI studies, the induced chronic tinnitus was accompanied by increased neuronal activity in the basolateral and cortical-like subdivisions of the amygdaloidal complex. We also found that blast trauma induced neuronal hyperactivity in the basolateral and cortical-like subdivisions of the amygdala and anterior cingulate cortex, and caused elevated anxiety. Evidence of increased synaptic activity in the amygdala and anterior cingulate cortex core suggests an association between central plasticity, blast-induced tinnitus, and symptoms of post-traumatic stress like anxiety. Anatomically, blast exposures induced TBI as demonstrated by sustained astrogliosis and prolonged axonal injury changes in auditory brain centers, suggesting that blast-induced tinnitus may predominately result from impact through the acoustic/auditory pathways. These findings also indicate that blast-induced tinnitus is potentially associated with traumatic brain injury, which may contribute to continued maladaptive neural plasticity. In addition, concussion may induce onset rather than chronic tinnitus and auditory detection deficits. In general, the induced effects are only accompanied by moderate level of axonal injury.

Human studies:

In individuals with blast induced hearing loss and tinnitus, we show significant relations among hearing, psychometric, neuropsychological variables and neuroimaging related parameters; most notably metabolites and neurotransmitters and white matter tracks vis-à-vis the FA metric. The association with SWI variables is less clear. Furthermore, these results suggest that several aspects of processing speed and delayed incidental recall during computer-based cognitive evaluation are associated with tinnitus severity. Specifically, variations in audiometric sensitivity appear to be related to performance accuracy, reaction time, and intra-individual variability on measures of processing speed and delayed-incidental recall. Our results for individuals with blast-induced hearing loss and tinnitus are consistent with prior research on patients with chronic tinnitus which imply that slowed reaction time, increased reaction time variability, and lower accuracy may be associated with difficulty inhibiting attention to distracting tinnitus signals.

## REFERENCES:


1. Gallyas F, Wolff JR, Bottcher H, Zaborszky L (1980) A reliable method for demonstrating axonal degeneration shortly after axotomy. Stain technology 55:291-297.
2. Kallakuri S, Cavanaugh JM, Ozaktay AC, Takebayashi T (2003) The effect of varying impact energy on diffuse axonal injury in the rat brain: a preliminary study. Exp Brain Res 148:419-424.
3. Kallakuri S, Singh A, Lu Y, Chen C, Patwardhan A, Cavanaugh JM (2008) Tensile stretching of cervical facet joint capsule and related axonal changes. European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society 17:556-563.
4. Kujawa SG, Liberman MC (2009) Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. J Neurosci 29:14077-14085.
5. Luo H, Zhang X, Nation J, Pace E, Lepczyk L, Zhang J (2012) Tinnitus suppression by electrical stimulation of the rat dorsal cochlear nucleus. Neurosci Lett 522:16-20.
6. Pace E, Zhang J (2013) Noise-induced tinnitus using individualized gap detection analysis and its relationship with hyperacusis, anxiety, and spatial cognition. PLoS One 8:e75011.

7. Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, Hughes LF, Caspary DM (2006) Gap detection deficits in rats with tinnitus: a potential novel screening tool. Behav Neurosci 120:188-195.
8. Waller HJ, Godfrey DA (1994) Functional characteristics of spontaneously active neurons in rat dorsal cochlear nucleus in vitro. Journal of neurophysiology 71:467-478.
9. Yang G, Lobarinas E, Zhang L, Turner J, Stolzberg D, Salvi R, Sun W (2007) Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. Hear Res 226:244-253.

**APPENDICES:**

Benson RR, Gattu R, Cacace AT. Left hemisphere fractional anisotropy increase in noise-induced tinnitus: A diffusion tensor imaging (DTI) study of white matter tracts in the brain. Hear Res (E-pub ahead of print)

Mahoney MJ, McFarland DJ, Carpenter MS, Rizvi S, Cacace AT. Reliability of broadband middle-ear power-reflectance in younger and older adults: Application of Generalizability Theory. Am J Audiol. (E-pub ahead of print)

Jessica Ouyang, Edward Pace, Laura Lepczyk, Michael Kaufman, Wei Zhang, and Jinsheng Zhang (being revised) Limbic involvement in blast-induced tinnitus, anxiety and related post-traumatic stress disorder in rats. *Experimental Neurology.*

Gulrez Mahmood, Zhigang Mei, Houmehr Hojjat, Edward Pace, Jinsheng Zhang (being reviesed) “Therapeutic Effect of Sildenafil on Blast-Induced Tinnitus and Auditory Impairment”, *Neuroscience*.

Hao Luo, Xueguo Zhang, Edward Pace and Jinsheng Zhang (being revised) Blast-Induced Tinnitus and Spontaneous Firing Changes in the Rat Dorsal Cochlear Nucleus, J. Neurosci Res.

AJA

Research Article

# Reliability of Broadband Middle-Ear Power Reflectance in Younger and Older Adults: Application of Generalizability Theory

Marty J. Mahoney,[a] Dennis J. McFarland,[b] MiChelle S. Carpenter,[a] Sabahet Rizvi,[a] and Anthony T. Cacace[a]

**Purpose:** To assess the reliability of broadband middle-ear power reflectance (BMEPR) and transmittance profiles for chirp and tonal stimuli using generalizability theory (GT).
**Method:** In adults without a history of middle-ear disease, the authors assessed the reliability of BMEPR to chirp and tonal stimuli using a multivariate approach based on an analysis of variance model (GT). For comparisons with other published studies, Pearson's product–moment correlation coefficients (Pearson's *r*) also were used.
**Results:** Based on GT with chirp stimuli, overall BMEPR measures had good reliability; however, the reliability of individual profiles across frequencies and ears was less than optimal. Lower generalizability coefficients were found when transmittance was evaluated. Test–retest reliability using Pearson's *r* was better for right versus left ears, and mid-frequencies were generally more reliable than those at either extreme of the frequency range. In contrast, tonal stimuli had higher generalizability coefficients and Pearson's *r* values than chirps for all frequencies tested; Pearson's *r* values were also higher for right versus left ears.
**Conclusion:** The authors extended the use of GT as a preferred way to evaluate reliability of BMEPR and transmittance profiles for chirps and tones because it allows for a more comprehensive evaluation compared with unidimensional pairwise correlations.

**Key Words:** adults, audiology, hearing, middle ear, power reflectance, generalizability theory

Measurement of broadband middle-ear power reflectance (BMEPR) represents an emerging technology for evaluating electroacoustic characteristics of human middle-ear function in vivo (Allen, Jeng, & Levitt, 2005; Jeng, Allen, Lapsey-Miller, & Levitt, 2008).[1] With this method, high-resolution frequency reflectance, absorbance, and/or transmittance profiles offer bio-inspired assessment opportunities for evaluating the middle ear under normal and pathological conditions (see, e.g., Feeney, Grant, & Marryott, 2003; Feeney, Grant, & Mills, 2009; Hunter, Tubaugh, Jackson, & Propes, 2008; Keefe & Simmons, 2003; Shahnaz, Bork, et al., 2009; Shahnaz, Longridge, & Bell, 2009). Nevertheless, as BMEPR measures transition from the laboratory to the clinic, the need for establishing the reliability of these measures is an important factor for test evaluation and clinical decision making.

Given the broadband characteristics of this metric, methodological and design considerations should take into account whether to base reliability on individual data points (frequencies; Hunter et al., 2008), select bands of frequencies (see, e.g., Beers, Shahnaz, Westerberg, & Kozak, 2010;

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[1]Broadband electroacoustic measures of middle-ear function can be represented in a number of different formats: power reflectance, absorbance, transmittance, and so on. *Middle-ear power reflectance* is defined as the ratio of reflected power to the incident power, which, when normalized, can range from 0 to 1 (where 0 = no reflectance and 1 = maximum reflectance), or it can be expressed as a percentage, from 0% to 100%. Absorbance is just a linear transformation of reflectance (1 – reflectance) representing the amount of energy that is absorbed versus reflected from the tympanic membrane/middle-ear system. Transmittance transforms the absorbance metric into a decibel scale (see Equation 1). Furthermore, there is no standard at present for representing how these measures should be expressed. To be clear, expressing these data as either a power reflectance or absorbance metric is a preference and not a requirement; it will not fundamentally change the result. It has been suggested that, by converting absorbance to transmittance, this metric would be less variable and may be more amenable to comparisons with hearing loss, because hearing loss is also expressed on a logarithmic (dB) scale (e.g., Allen et al., 2005; Jeng et al., 2008; Keefe & Simmons, 2003).

***Disclosure:*** *The authors have declared that no competing interests existed at the time of publication.*

Shahanaz, Bork, et al., 2009; Vander Werff, Prieve, & Georgantas, 2007), or the shape of the entire frequency reflectance profile (the present study). Moreover, special consideration should be given as to whether to compute reliability metrics based on absolute difference measures of central tendency and dispersion (*M*s and *SD*s), Pearson product–moment correlation coefficients (Pearson's *r*), or generalizability theory (GT).

Although researchers have used different approaches to assess test–retest reliability, some methods are on more secure scientific ground than others. For example, absolute differences of BMEPR have been used as indices of test–retest reliability when values are measured over two or more points in time (see, e.g., Beers et al., 2010; Rosowski et al., 2012; Shahnaz, Bork, et al., 2009; Vander Werff et al., 2007; Werner, Levi, & Keefe, 2010). On the surface, this approach makes intuitive sense because a reliable test is one that produces similar results on two different occasions. However, with the absolute-difference method, the interpretation of good or poor reliability is unduly subjective. This is due to the fact that this method does not take variability into account, and its meaning depends on the scale of measurement applied. In contrast, the more conventional Pearson's *r* provides a standardized metric for reliability calculations because it is based on the proportion of variance that is repeatable. Because Pearson's *r* represents a normalized difference metric that is signed, it allows for direct comparisons to be made with other studies because it is independent of the unit of measurement (see, e.g., Hunter et al., 2008; Werner et al., 2010; the present investigation). Although the advantages of using Pearson's *r* over the absolute-difference method are apparent, this approach is limited to pairwise comparisons; therefore, the univariate nature of this metric is not well suited for complex data sets. In comparison to these latter two measures, GT is a multivariate approach to reliability based on an analysis of variance (ANOVA) model, in which more than two points in time and multiple independent variables can be jointly considered in the computations. Furthermore, GT is unique when compared with the typical ANOVA model. Whereas the typical ANOVA model considers subjects as the source of error and considers trends over time as the effect of interest, in GT, the variance associated with subjects is the effect of interest, and the variance over time is the source of error. With this strategy, the resultant generalizability coefficient becomes a measure of the effect size (i.e., the size of the main effect for subjects) that represents the proportion of variance that is due to consistent individual differences. Thus, GT provides a framework for assessing multiple time points, including main effects and interactions between multiple independent variables. Of particular relevance to the current area of interest is the interaction between subjects and stimulus frequency because this relationship allows for the reliability of individual frequency reflectance profiles to be assessed.

It is our contention that the choice and rationale of whether to base reliability estimates on individual data points, on select bands of frequencies, or on profiles should depend on how clinicians actually use these measurements to diagnose middle-ear disorders. For example, if a diagnosis is based on a single point or on a single band, independent of the overall shape of the profile, then the reliability of individual data points or bands would be the appropriate index. However, as Keefe and Simmons (2003) noted,

> There is no evidence to suggest that the use of a single frequency, as in clinical tympanometry, is optimal for assessing middle-ear function at all frequencies important in auditory communication systems, no more than would a single frequency suffice for assessing cochlear, behavioral, or neural function. Wideband measurements of middle-ear functioning appear to have promise as a clinical diagnostic test. (p. 3217)

In this article, we extend the logic of Keefe and Simmons to include the fact that if clinicians base their diagnosis on the shape of the entire profile, then the reliability of the profile would be the most appropriate feature to evaluate. We focus herein on the use of GT for establishing test–retest reliability of BMEPR data, whereby the effects of multiple variables are to be considered (see, e.g., Crocker & Algina, 1986; Cronbach, Nageswari, & Gleser, 1963; Laenen, Vangeneugden, Geys, & Molenberghs, 2006). Last, because GT has not been used extensively in the audiological/hearing science literature, we provide a concise overview to acquaint readers with this topic (see the Appendix).

## Method and Materials

Fifty-six adults, categorized into two age groups (Group 1: 18–25 years, $n = 28$; Group 2: $\geq 50$ and $\leq 66$ years, $n = 28$), were studied. Each age group was stratified by gender (14 men, 14 women) and ear (56 left, 56 right) and, therefore, provided a balanced design among age group, gender, ear, and frequency. Because subjects were recruited by word of mouth from friends, relatives, and students, the data obtained were considered a convenience sample. Inclusion criteria were a negative history of middle-ear disease; no air–bone gaps exceeding 10 dB for any frequency; and ear canals free of obstruction or debris, based on a screening otoscopic exam. The Human Investigation Committee at Wayne State University approved this study, and we obtained signed informed consent from each individual prior to data collection.

Audiometric testing was conducted in a commercial sound booth (Acoustic Systems, Model RE-144) through use of a clinical audiometer (Grason-Stradler, Model 61) with standard earphones (Telephonics, Model TDH-50P) enclosed in supra-aural ear cushions (MX-41/AR). Pure-tone air-conduction audiometry was performed at octave frequencies from 250 Hz through 8000 Hz and at one-half–octave frequency (3000 Hz) bilaterally. Bone-conduction testing used a standard oscillator (Radioear B-71) and a standard headband. Bone-conduction thresholds were assessed at octave frequencies ranging from 250 Hz through 4000 Hz.

BMEPR was measured using commercially available hardware and software (Mimosa Acoustics, MEPA3 Clinical

Reflectance System) and a high-quality probe assembly (Etymotics, Model ER10C) to transduce acoustic stimuli and record acoustic responses from the ear canal. Before each recording session, the MEPA3 system was successfully calibrated in a four-chamber coupler (Model CC4-V) in accordance with guidelines provided by the manufacturer. Particular care was taken to ensure that the foam ear tip of the probe was properly seated and stable in the ear canal. There were no crimps in the foam, and this coupling device was fully expanded in the ear canal before testing was initiated. After measurements from chirp and tonal stimuli were obtained from each ear, the probe was removed and reinserted into the same ear canal, and a second set of chirp and tonal measures was acquired. Then, the second ear was tested using the same approach. For the present investigation, both sets of within-session data were used in this analysis. The SPL of the chirp stimulus was set to 60 dB (re: 20 uPa), and data were collected over a 1-s time epoch at ambient ear canal air pressure. This allowed for 24 individual chirps (~5 ms in total duration) to be collected and averaged. The SPL of the tonal stimuli was also set to 60 dB (re: 20 uPa), and nine individual pure tones (ranging from 257 Hz to 6000 Hz) were analyzed. Individual tonal stimuli were 300 ms in total duration, presented sequentially from low to high frequency and separated by a 150-ms silent interstimulus interval. Selection of the initial ear of measurement was randomized by a physical coin toss (heads = left ear, tails = right ear) to avoid potential order effects that might confound data interpretation (see, e.g., Thornton, Marotta, & Kennedy, 2003). The same medium-sized foam ear tips (14A) were used during instrument calibration and data collection. With respect to chirp stimuli, out of a possible 248 frequencies measured, we selectively sampled a subset of 16 frequencies (258, 307, 398, 492, 633, 750, 796, 1008, 1270, 1500, 1590, 1992, 2530, 3000, 4060, and 5040 Hz) for this investigation that clearly outlined the frequency reflectance profile. With respect to tonal stimuli, we used default values and sampled nine separate frequencies (258, 492, 750, 1007, 1500, 1992, 3000, 4007, and 6000 Hz). Power-reflectance values associated with both chirp and tonal stimuli were extracted from separate stored output files that were available from each subject.

To allow for reliability to be evaluated from a multivariate perspective, we conducted an ANOVA to compute the generalizability coefficients. To allow for comparisons with other published studies in the literature, we also used Pearson's *r* to evaluate test–retest reliability for individual frequencies.

## Results

Figure 1 shows grand averaged frequency reflectance profiles separately for chirp and tonal stimuli collapsed across all variables and for all combinations of age, gender, and ear variables. Except for the highest frequency studied, average power-reflectance values corresponding to each stimulus type (chirp and tone) were very similar. In Figure 2, individual scatter plots are shown for 16 frequencies and all test–retest conditions for chirp stimuli. The general trends observed in these plots show that within-session variability increased from low to high frequencies. Figure 3 shows individual scatter plots for nine separate tonal frequencies and for all test–retest conditions. Although a similar trend for increased within-session variability with increases of stimulus frequency was also observed, tonal stimuli showed less within-session variability than did chirps.

For comparison with previous studies, in Table 1 (left side) we provide Pearson's *r* values for the test–retest reliability of 16 individual frequencies for chirp stimuli, separately for each ear. Trends in these data reveal higher reliabilities for right versus left ears, with mid-frequency reliabilities generally higher than those at either extreme. Test–retest reliabilities of Pearson's *r* values for the nine individual frequencies are presented in the right side of Table 1, separately for each ear obtained for tonal stimuli. These data show higher test–retest correlation values in comparison to chirps at corresponding frequencies; right ears also showed higher test–retest correlations than left ears.

Next, we analyzed both chirp and tonal data sets with an ANOVA, in two ways. First, the effect of subjects was used in the error terms to evaluate the consistency of age, gender, ear, and test effects across subjects (i.e., traditional null hypothesis significance testing). The second set of analyses used tests in the error terms to evaluate the proportion of variance due to subject effects that was consistent across tests (i.e., generalizability or test–retest reliability). Results were calculated for both power reflectance and transmittance. We analyzed the transmittance metric because we thought that this transformation might reduce variability and thus potentially improve the generalizability coefficients (Allen et al., 2005), keeping in mind that although this assertion was suggested by Allen and colleagues (2005), it has never been proven empirically.

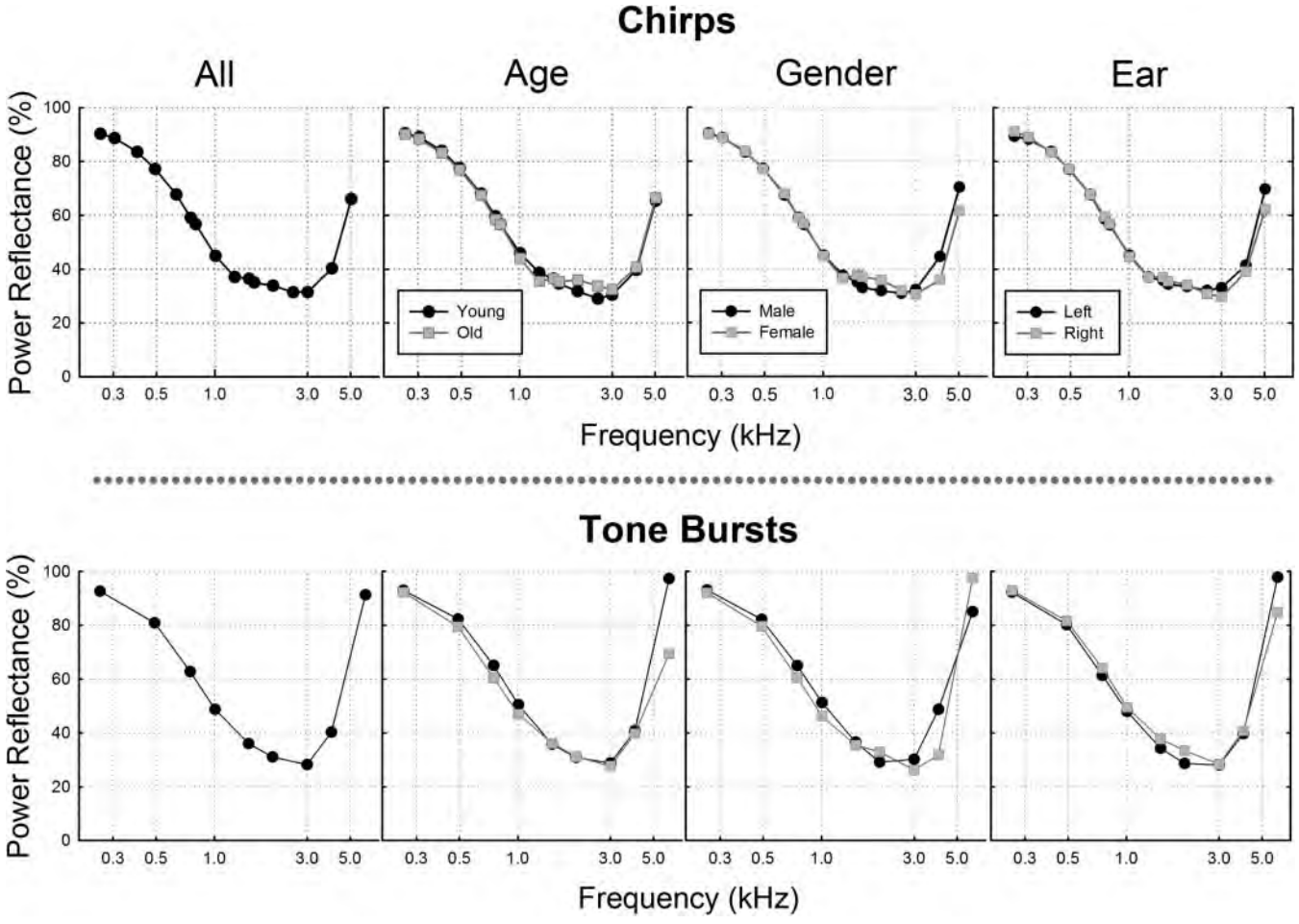
### *Chirps*

We conducted a six-way ANOVA in which the effects of age and gender were used as between-subjects variables and ear, frequency, and time were used as within-subject variables. With subject effects used as the error term, the ANOVA showed a significant main effect of frequency ($F = 350.31$, $p < .0001$), resulting from the lower power reflectance in mid-frequencies, as seen in all plots of Figure 1. There were also Gender × Ear ($F = 4.22$, $p < .045$), Gender × Frequency ($F = 2.38$, $p < .002$), and Age × Gender × Ear ($F = 4.60$, $p < .037$) interactions. The Gender × Frequency interaction was due in part to greater reflectance in men at the highest frequencies. The three-way interaction was associated with greater reflectance in the right ear of older women and the left ear of older men, with less difference in younger women and men.

The results of the ANOVA in which test effects were used as the error term resulted in the generalizability coefficients shown in Table 2 (left side). Also shown in Table 2 (right side) are generalizability coefficients for the transmittance values, computed as follows:

$$T = 10 \times \log_{10}[1 - (|R|^2)](\mathrm{dB}), \quad (1)$$

**Figure 1.** Grand averaged frequency–reflectance profiles for chirps and tonal stimuli. The demarcations noted at the top of each figure ("All," "Age," "Gender," "Ear") represent individual categories of data. "All" indicates that data were collapsed across age, gender, and ear; "Age" (young and old) indicates that data were collapsed across gender and ear; "Gender" (male and female) indicates that data were collapsed across age and ear; and "Ear" (left and right) indicates that data were collapsed across age and gender.




where $T$ is the transmittance and $|R|^2$ is the power reflectance expressed as a proportion in decibels.

These data show that the generalizability coefficient associated with the main effect of subjects was 0.82. This score represents the average for each subject collapsed over frequency and ear. The reliability associated with the Subjects × Frequency interaction was 0.56. This effect corresponds to the profile for individual subjects across frequencies averaged across both ears. The reliability of the Subjects × Ear × Frequency interaction was 0.35. This corresponds to the shape of the profile for individual subjects across frequencies for individual ears and probably represents the feature of greatest interest to the clinician. Generalizability coefficients for transmittance were somewhat lower, particularly for the three-way interaction.

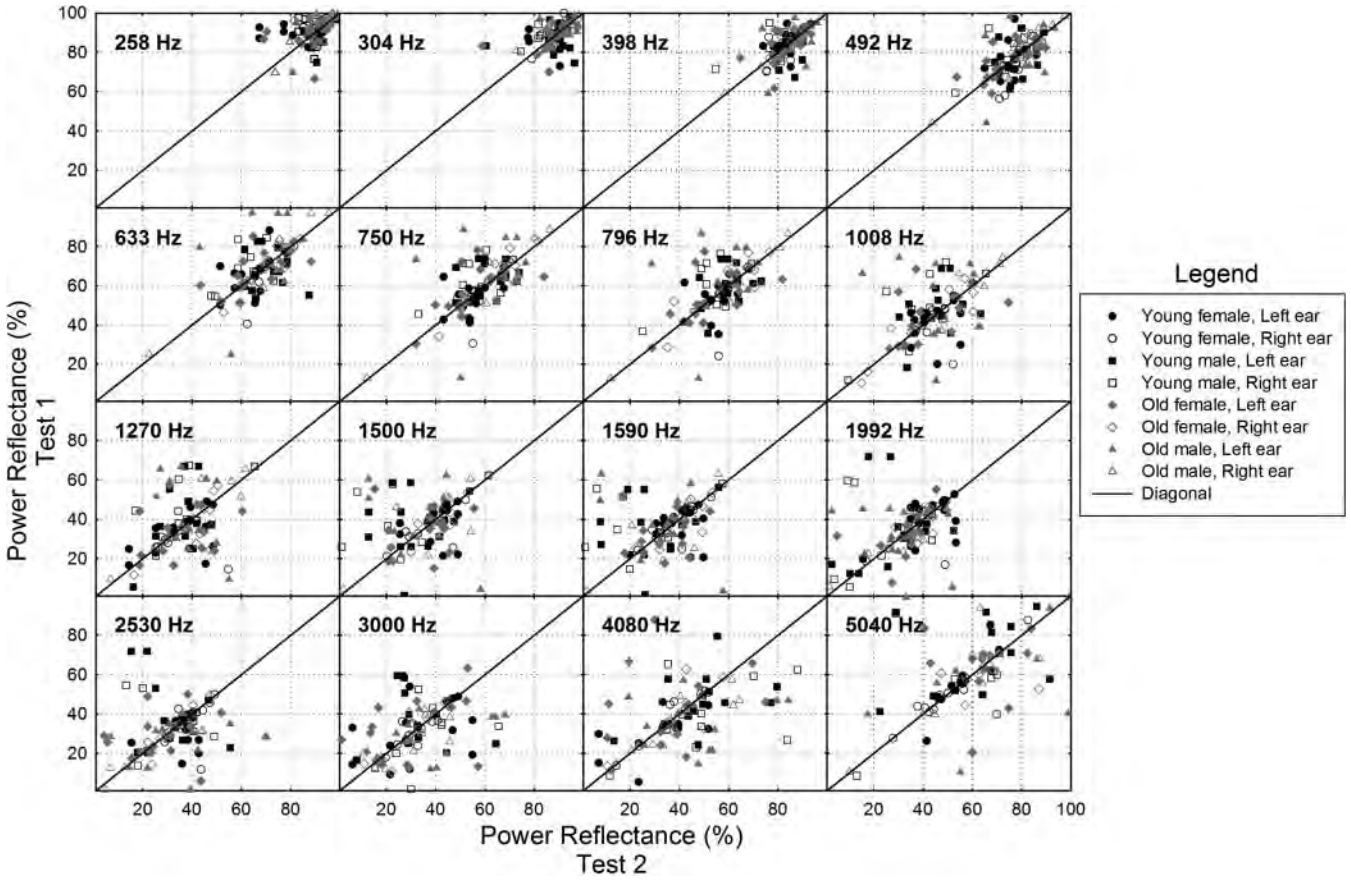
### *Tonal Stimuli*

Generalizability coefficients for middle-ear power reflectance using tonal stimuli are shown in Table 3 (left side). The generalizability coefficient associated with the main effects of subjects was 0.86. The reliability associated with the Subjects × Frequency interaction was 0.72. The reliability associated with the Subjects × Ear interaction was 0.75, and that for the Subjects × Ear × Frequency interaction was 0.63. Because reliability coefficients are correlations, differences can be evaluated with Fisher's $z$ transformation, and significance levels depend on the number of cases studied. In the present comparison (0.63 vs. 0.75), in which there were 56 subjects, the difference was not significant (see Ramseyer, 1979). Nevertheless, these values were considerably higher than those reported for chirps. Values for the transmittance data for tonal stimuli (see Table 3, right side) were similar to the power-reflectance data.

## Discussion

Cronbach and colleagues (1963) and Cronbach, Gleser, Nanda, and Rajaratnam (1972) initially introduced GT

**Figure 2.** Composite scatter plots for 16 frequencies for different variables studied. Data collected from Test 1 are plotted on the *y*-axis, and data collected from Test 2 are plotted on the *x*-axis. If the within-session data from Test 1 and Test 2 were identical for each of the different frequencies, then data points would fall directly on the solid diagonal line in each of the plots. On the basis of the scatter of data points observed, the degree of within-session variability appears rank ordered from low, to middle, to higher frequencies.




into the educational psychology literature to evaluate the reliability of profiles of standardized test scores in school-age children. Because of its unique capabilities of assessing reliability from a multivariate perspective, GT has garnered increased interest in other fields of inquiry, including speech, hearing, vestibular, and physical sciences, where a wide range of topics—physiology, electroacoustics, perception, responses to questionnaires, and so forth—have already been investigated. Relevant examples include the reliability of distortion product otoacoustic emissions over a 24-hr time period (Cacace, McClelland, Weiner, & McFarland, 1996), postural control in the evaluation of concussion (Broglio, Zhu, Sopiarz, & Oark, 2009), speechreading abilities (Demorest & Bernstein, 1992), perceptual scaling (S. O'Brian, Packman, Onslow, & O'Brian, 2003), analysis of observational data (N. O'Brian, O'Brian, Packman, & Onslow, 2003; Scarsellone, 1998), videographic representation of tooth and lip position in smiling and speech following orthodontic and dentofacial surgery (van de Geld, Oosterveld, van Waas, & Kuijpers-Jaqtman, 2007), and in force measurements used in physical therapy and rehabilitation (Roebroeck, Hariaar, & Lankhors, 1993). Use of GT in assessing the reliability of BMEPR profiles expands this list of testing domains to include another form of auditory-based electroacoustic analysis, in which profiles involving multiple frequencies evaluated at two or more points in time and numerous independent variables (age, gender, and ear) are under consideration.

We chose to analyze power-reflectance profiles of individual frequencies as our primary metric because this is the relevant feature derived from commercially available instrumentation and the one that clinicians would actually use to make inferences about normal or pathological states of the middle ear. Moreover, advanced textbooks on research design and statistics consider GT the most comprehensive technique available for estimating test measurement reliability (Schiavetti & Metz, 2006, p. 123), and they do not even recognize the absolute-difference method, as described in this article, as a metric of reliability (Maxwell & Satake, 2006).

Established diagnostic exemplars of this methodology include categories of tympanometric types (i.e., profiles of immittance shape as a function of positive and negative air pressures; see, e.g., Jerger, Jerger, & Mauldin, 1972) or on

**Figure 3.** Composite scatter plots for nine separate tonal frequencies for different variables studied. Data collected from Test 1 are plotted on the *y*-axis, and data collected from Test 2 are plotted on the *x*-axis.

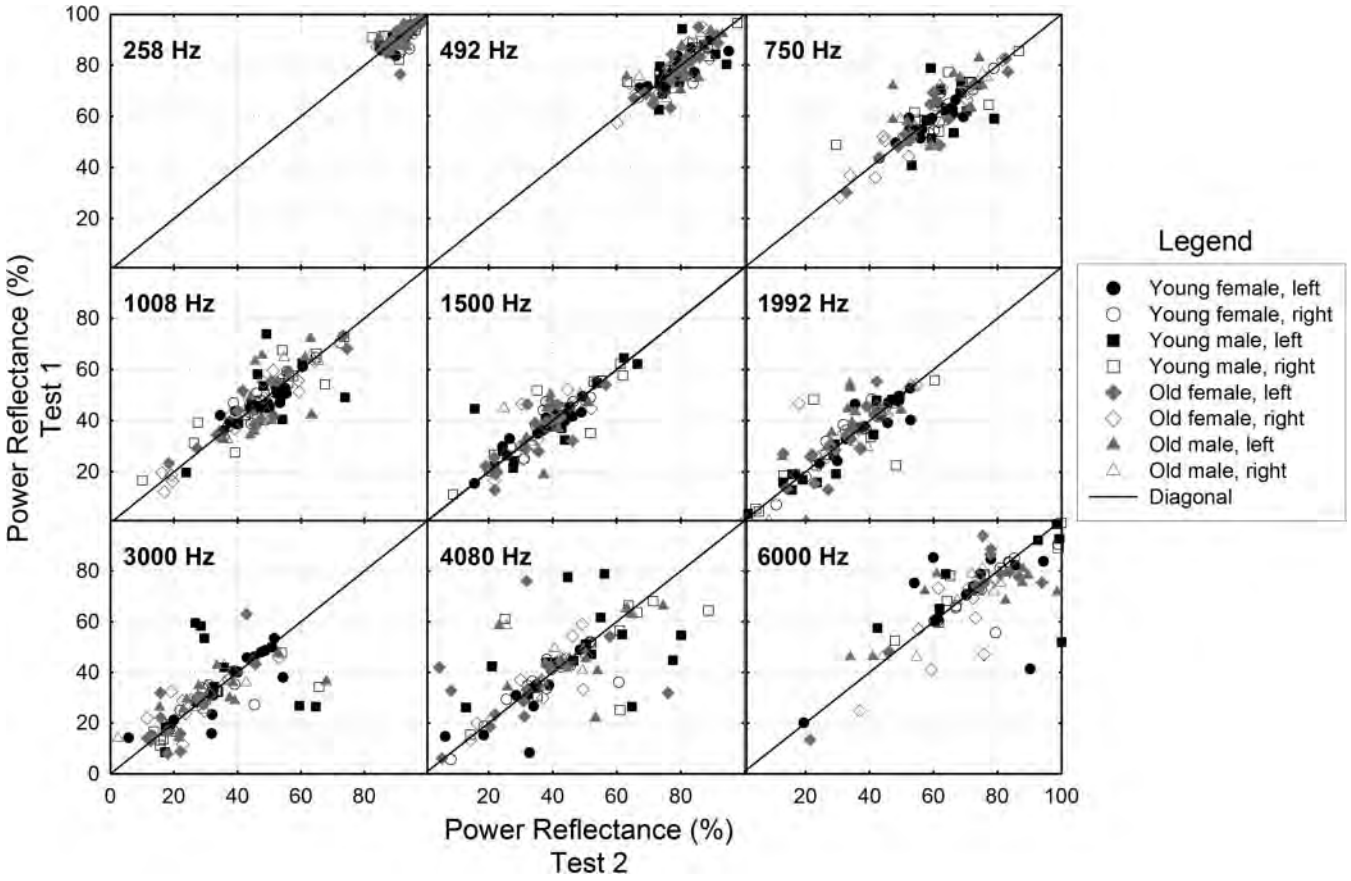



**Table 1.** Pearson's *r* correlations for chirps and tones.

| | Chirps | | Tones | |
|---|---|---|---|---|
| Frequency (Hz) | Left ear | Right ear | Left ear | Right ear |
| 257 | 0.24 | 0.50** | 0.75** | 0.66** |
| 304 | 0.17 | 0.70** | | |
| 398 | 0.44** | 0.83** | | |
| 492 | 0.52** | 0.83** | 0.78** | 0.83** |
| 632 | 0.50** | 0.85** | | |
| 750 | 0.60** | 0.85** | 0.82** | 0.87** |
| 796 | 0.57** | 0.82** | | |
| 1007 | 0.53** | 0.81** | 0.82** | 0.92** |
| 1265 | 0.44** | 0.69** | | |
| 1500 | 0.30* | 0.60** | 0.52** | 0.85** |
| 1593 | 0.34** | 0.64** | | |
| 1992 | 0.55** | 0.61** | 0.58** | 0.87** |
| 2531 | 0.31* | 0.63** | | |
| 3000 | 0.38** | 0.73** | 0.67** | 0.84** |
| 4007 | 0.51** | 0.66** | 0.64** | 0.78** |
| 5039 | 0.26 | 0.72** | 0.79 | 0.89** |

*$p < .05$. **$p < .01$.

more quantitative immittance typologies based on the theoretical model of Vanhuyse (see Margolis, Van Camp, Wilson, & Creten, 1985; Van Camp, Margolis, Wilson, Creten, & Shanks, 1986; Vanhuyse, Creten, & Van Camp, 1975). The potential for BMEPR measures to identify and delineate different pathological conditions of the middle ear provides the rationale and support for instituting a profile analysis because diagnostic interpretations are already being made using this approach (see, e.g., Allen et al., 2005; Feeney et al., 2003, 2009; Keefe & Simmons, 2003; Shahnaz, Longridge, & Bell, 2009).

Based on GT using chirps, the reliability for the overall effect (i.e., the power reflectance averaged across all frequencies) was 0.82; this is acceptable by standard convention (Cicchetti, 1994). The reliability of the frequency reflectance profile (the shape of the profile independent of height) was 0.56, which would be considered fair. Repeating the test and averaging the results yielded a profile reliability of 0.72, which is considered good. However, profiles involving ears and Ear × Frequency interactions were at levels conventionally considered to be poor (0.35). For tonal stimuli, the reliability of the overall effect was 0.86. The reliability of the frequency

**Table 2.** Chirp generalizability for reflectance and transmittance.

| Effect/error | Reflectance | | | Transmittance | | |
|---|---|---|---|---|---|---|
| | MS | $\rho^2$ | *SE* | MS | $\rho^2$ | *SE* |
| Subjects | 2,360.880 | 0.8196 | 0.0241 | 55.95144 | 0.7788 | 0.0296 |
| Subjects × Time | 260.713 | | | 6.95624 | | |
| Subjects × Frequency | 291.408 | 0.5621 | 0.0585 | 18.49421 | 0.4771 | 0.0699 |
| Subjects × Frequency × Time | 81.681 | | | 6.54339 | | |
| Subjects × Ear | 609.427 | 0.3877 | 0.0818 | 11.19549 | 0.2581 | 0.0991 |
| Subjects × Ear × Time | 268.925 | | | 6.60240 | | |
| Subjects × Ear × Frequency | 108.633 | 0.3484 | 0.0871 | 6.98064 | 0.0133 | 0.1319 |
| Subjects × Ear × Frequency × Time | 52.494 | | | 6.79778 | | |

*Note.* MS = mean square; $\rho^2$ = generalizability coefficient; *SE* = standard error.

reflectance profile was 0.72. Repeating the test and averaging the results yielded a profile reliability of 0.75, and profiles involving ears and Ear × Frequency interactions were at 0.63. Thus, in comparison to chirps, the reliability for tones was better.

Using chirps, we made within-session test–retest reliability measures using Pearson's *r* for 16 individual frequencies ranging from 258 Hz to 5040 Hz. These data, which were collapsed across gender and age group, ranged from 0.30 to 0.85 for the right ear and from 0.18 to 0.57 for the left ear. These values agreed favorably with the adult data of Werner et al. (2010), who assessed 15 frequencies across a similar bandwidth (ranging from 281 Hz to 7336 Hz). Their correlation values from adults ranged from 0.28 to 0.95, where data were collapsed across gender and were presented for right ears only. In addition, Hunter and colleagues (2008) provided test–retest correlation coefficients for nine frequencies, ranging from 258 Hz to 6000 Hz, with data collapsed across age, gender, and ear and focused on children ranging in age from 3 days to 47 months. Their correlation values ranged from 0.68 to 0.97 and were higher than those reported in adults. Test–retest correlation values for tonal stimuli from the present study ranged from 0.52 to 0.92.

Werner and colleagues (2010) used a hybrid approach to assess reliability of power reflectance in infants and adults using three different metrics: (a) absolute-difference measures, (b) test–retest correlations of 15 individual frequency bands using Pearson's *r,* and (c) the cross-correlation method to examine reliability of the entire profile for the same ear and for left and right ears on two occasions. In their study, test–retest correlations for individual frequency bands were predominantly positive and were statistically significant. The highest correlation values were generally observed in the lower frequency range; the lowest correlations were observed in the higher frequency range. When test–retest correlations were averaged across frequency for the individual age groups (our computations are based on Table 1 of Werner et al., 2010), they were rank ordered, being lowest for 5- to 9-month-olds ($M$ = 0.274, $SD$ = 0.158, range: –0.05 to 0.44), intermediate for 2- to 3-month-olds ($M$ = 0.401, $SD$ = 0.139, range: 0.16 to 0.57), and highest for adults ($M$ = 0.551, $SD$ = 0.196, range: 0.30 to 0.95). In regard to adults, the cross-correlation method that was used to assess reliability of the shape of the profile and collapsed across age group produced a value of 0.85; the average between-ear cross-correlation was 0.84. However, it is noteworthy that the cross-correlation statistic is typically performed by comparing two time series, using a lag term to shift one function against the other as a way to determine the maximum correlation. Werner and colleagues indicated that "frequency was the lag variable" (p. 7), but they did not elaborate on the nonconventional use of this statistic. Applying the cross-correlation in this way results in profiles being aligned at different frequencies, a practice that contrasts with typical usage and one that has a questionable theoretical rationale because direct comparisons across frequency are not possible. Nevertheless, it is our contention

**Table 3.** Tone generalizability for reflectance and transmittance.

| Effect/error | Reflectance | | | Transmittance | | |
|---|---|---|---|---|---|---|
| | MS | $\rho^2$ | *SE* | MS | $\rho^2$ | *SE* |
| Subjects | 988.5041 | 0.8546 | 0.0194 | 10.39252 | 0.8505 | 0.0200 |
| Subjects × Time | 77.5189 | | | 0.83962 | | |
| Subjects × Frequency | 258.4342 | 0.7218 | 0.0372 | 3.06893 | 0.7888 | 0.0282 |
| Subjects × Frequency × Time | 41.7633 | | | 0.36238 | | |
| Subjects × Ear | 321.4741 | 0.7541 | 0.0315 | 4.30111 | 0.7114 | 0.0386 |
| Subjects × Ear × Time | 42.9896 | | | 0.72534 | | |
| Subjects × Ear × Frequency | 100.8526 | 0.6296 | 0.0495 | 0.83801 | 0.5346 | 0.0622 |
| Subjects × Ear × Frequency × Time | 22.9267 | | | 0.25412 | | |

that the overall shape of the profile (e.g., power-reflectance values across frequencies) within individual ears is the primary feature of interest to clinicians using it for diagnostic purposes.

As noted above, we found that the reliability estimates of BMEPR and transmittance values were better for tones than for chirps. Although the precise reason for this disparity remains to be determined, several factors can be investigated in future studies. Consider that a broadband chirp is a continuously changing dynamic waveform in the time domain that is spectrally complex in the frequency domain. Even though the input waveform has a short duration (~5 ms), it is evaluated over a longer time epoch (1 s), such that acoustic reflections from the eardrum can be captured by the probe microphone, allowing for computations to be made on the metric of interest. With this in mind, single cycles of individual frequencies may be susceptible to interference and/or perturbations from physiologic events that may be present in a closed ear canal, such as respirations, blood flow pulsations from surface vessels, spontaneous or evoked otoacoustic emissions from the cochlea, subject movement, cord noise, and so forth. Thus, we speculate that, either alone or in combination, these factors can lead to increased variability in measurement. On the other hand, individual tonal stimuli are repetitive steady-state oscillations and perhaps are less affected by physiologic (state) and subject (trait) variables.

Ear-specific profiles of measurements across frequencies are commonplace in the field of diagnostic audiology; obvious examples include audiograms, iso-level/frequency profiles of distortion-product otoacoustic emissions (aka DPGrams), tympanograms, and BMEPR profiles. Thus, the problem of assessing reliability is ubiquitous in this field, and GT provides a robust solution to this problem. As noted above, we have already applied GT to DPGrams and evaluated the influences of time of day, stimulus frequency, stimulus SPL, and gender in adults with normal hearing (Cacace et al., 1996). We found that DPGrams were reliable measures within subjects over a contiguous 24-hr time period. Significant and reliable differences and interactions across frequency, SPL, and gender were also observed.

Another issue that requires further study concerns which characteristics of a profile might be meaningful clinically and how clinicians and researchers could potentially use this information to improve reliability estimates. As an example, Feeney et al. (2003) and Shahnaz, Bork, et al. (2009) have described trends associated with middle-ear pathology as alterations in broadband features (e.g., increased low-frequency power reflectance associated with otosclerosis). Because broadband changes such as those seen in otosclerosis involve lower-order trends, we speculate that it might be useful to fit these profiles with simple functions that capture these trends and smooth over measurement noise. Therefore, trend analysis might be a useful way to model these effects and, thereby, improve reliability. However, whether all clinically relevant information would be captured in lower-order trends remains to be seen; we suspect that this would not be the case. One alternative is to consider the possibility that the variability of a profile—made multiple times in the same individual—might represent a pathologic feature. In this context, it would be interesting to determine whether high variance in the residual after the removal of lower-order trends would be associated with clinically useful information. Pathological states of the middle ear that might show such effects include tympanic membrane perforations, monomeric tympanic membranes secondary to healed perforations, and ossicular discontinuities. Thus, further work in this area will be necessary to assess this hypothesis.

On the basis of the arguments presented in this article, and with respect to current clinical usage, profile analysis of BMEPR values appears to be the most relevant metric for evaluating and diagnosing middle-ear disorders. In contrast to tympanometry, which typically uses only one (226 Hz) or perhaps only a few specific probe-tone frequencies (e.g., 226 Hz, 660 Hz, 1200 Hz) to estimate characteristics of middle-ear function, power-reflectance techniques can rapidly measure hundreds of points over a much broader bandwidth and in a considerably shorter period of time (seconds). This is noteworthy because it allows for a more comprehensive account of energy transfer characteristics of the middle ear than is currently available from other methods. Combined with computer-controlled hardware and based on rapidity of measurement, BMEPR has many desirable attributes consistent with a viable clinical tool. Therefore, improving reliability of measurement is an essential requisite in the evolution of this method if it is to transition effectively from the laboratory to the clinic.

In conclusion, the reliability of BMEPR measurements is an important consideration in establishing this method for clinical decision making. The application of GT allows for a more comprehensive evaluation of these types of data compared with other approaches, and we advocate for the strategic use of this metric in future investigations.

## Acknowledgments

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## References

**Allen, J. B., Jeng, P. S., & Levitt, H.** (2005). Evaluation of human middle ear function via an acoustic power assessment. *Journal of Rehabilitation Research and Development, 42,* 63–78.

**Beers, A. N., Shahnaz, N., Westerberg, B. D., & Kozak, F. K.** (2010). Wideband reflectance in normal Caucasian and Chinese school-aged children and in children with otitis media with effusion. *Ear and Hearing, 31,* 221–233.

**Broglio, A. T. C., Zhu, W., Sopiarz, K., & Oark, Y.** (2009). Generalizability theory analysis of balance error scoring system reliability in healthy young adults. *Journal of Athletic Training, 44,* 497–502.

**Cacace, A. T., McClelland, W. A., Weiner, J., & McFarland, D. J.** (1996). Individual differences and the reliability of 2F1–F2 distortion-product otoacoustic emissions: Effects of time-of-day, stimulus variables, and gender. *Journal of Speech and Hearing Research, 39,* 1138–1148.

**Cicchetti, D. V.** (1994). Guidelines, criteria, and rules of thumb for evaluating normed and standardized assessment instruments in psychology. *Psychological Assessment, 6,* 284–290.

**Crocker, L., & Algina, J.** (1986). *Introduction to classical and modern test theory*. New York, NY: Harcourt Brace.

**Cronbach, L. J., Gleser, G. C., Nanda, H., & Rajaratnam, N.** (1972). *The dependability of behavioral measurements: Theory of generalizability for scores and profiles*. New York, NY: Wiley.

**Cronbach, L. J., Nageswari, R., & Gleser, G. C.** (1963). Theory of generalizability: A liberation of reliability theory. *British Journal of Statistical Psychology, 16,* 137–163.

**Demorest, M. E., & Bernstein, L. E.** (1992). Sources of variability in speech reading sentences: A generalizability analysis. *Journal of Speech and Hearing Research, 35,* 876–891.

**Di Nocera, F., Ferlazzo, F., & Borghi, V.** (2001). G theory and the reliability of psychophysiological measures: A tutorial. *Psychophysiology, 38,* 796–806.

**Feeney, M. P., Grant, I. L., & Marryott, L. P.** (2003). Wideband energy reflectance measurements in adults with middle-ear disorders. *Journal of Speech, Language, and Hearing Research, 46,* 901–911.

**Feeney, M. P., Grant, I. L., & Mills, D. M.** (2009). Wideband energy reflectance measurements of ossicular chain discontinuity and repair in human temporal bone. *Ear and Hearing, 30,* 391–400.

**Hunter, L. L., Tubaugh, L., Jackson, A., & Propes, S.** (2008). Wideband middle ear power measurement in infants and children. *Journal of the American Academy of Audiology, 19,* 309–324.

**Jeng, P. S., Allen, J. B., Lapsey-Miller, J. A., & Levitt, H.** (2008). Wideband power reflectance and power transmittance as tools of assessing middle-ear function. *Perspectives on Hearing and Hearing Disorders in Children, 18,* 44–57.

**Jerger, J., Jerger, S., & Mauldin, L.** (1972). Studies in impedance audiometry: Normal and sensorineural ears. *Archives of Otolaryngology, 96,* 513–523.

**Keefe, D. H., & Simmons, J. L.** (2003). Energy transmittance predicts conductive hearing loss in older children and adults. *The Journal of the Acoustical Society of America, 114,* 3217–3238.

**Laenen, A., Vangeneugden, T., Geys, H., & Molenberghs, G.** (2006). Generalized reliability estimation using repeated measures. *British Journal of Mathematical and Statistical Psychology, 59,* 113–131.

**Margolis, R. H., Van Camp, K. J., Wilson, R. H., & Creten, W. L.** (1985). Multifrequency tympanometry in normal ears. *Audiology, 24,* 44–53.

**Maxwell, D. L., & Satake, E.** (2006). *Research and statistical methods in communication sciences and disorders*. Boston, MA: Thomson Delmar Learning.

**Mushquash, C., & O'Connor, B.** (2006). SPSS and SAS programs for generalizability theory analysis. *Behavioral Research Methods, 38,* 542–547.

**O'Brian, N., O'Brian, S., Packman, A., & Onslow, M.** (2003). Generalizability theory I: Assessing reliability of observational data in the communication sciences. *Journal of Speech, Language, and Hearing Research, 46,* 711–717.

**O'Brian, S., Packman, A., Onslow, M., & O'Brian, N.** (2003). Generalizability theory II: Application to perceptual scaling of speech naturalness in adults who stutter. *Journal of Speech, Language, and Hearing Research, 46,* 718–723.

**Ramseyer, G. C.** (1979). Testing the difference between dependent correlations using Fisher *z*. *Journal of Experimental Education, 47,* 307–310.

**Roebroeck, M. E., Hariaar, J., & Lankhors, G. J.** (1993). The application of generalizability theory to reliability assessment: An illustration using isometric force measurements. *Physical Therapy, 73,* 386–395.

**Rosowski, J. J., Nakajima, H. H., Hamade, M. A., Mahfoud, L., Merchant, G. R., Halpin, C. F., & Merchant, S. N.** (2012). Ear-canal reflectance, umbo velocity, and tympanometry in normal-hearing adults. *Ear and Hearing, 33,* 19–34.

**Scarsellone, J. M.** (1998). Analysis of observational data in speech and language research using generalizability theory. *Journal of Speech, Language, and Hearing Research, 41,* 1341–1347.

**Schiavetti, N., & Metz, D. E.** (2006). *Evaluating research in communicative disorders* (5th ed.). Boston, MA: Pearson Education.

**Shahnaz, N., Bork, K., Polka, L., Longridge, N., Bell, D., & Westerberg, B. D.** (2009). Energy reflectance and tympanometry in normal and otosclerotic ears. *Ear and Hearing, 30,* 219–233.

**Shahnaz, N., Longridge, N., & Bell, D.** (2009). Wideband energy reflectance patterns in preoperative and post-operative otosclerotic ears. *International Journal of Audiology, 48,* 240–247.

**Thornton, A. R. D., Marotta, N., & Kennedy, C.** (2003). The order of testing effect in otoacoustic emissions and its consequences for sex and ear differences in neonates. *Hearing Research, 184,* 123–130.

**Van Camp, K. J., Margolis, R. H., Wilson, R. H., Creten, W. L., & Shanks, J. E.** (1986). Principles of tympanometry. *American Speech and Hearing Association Monographs, 24,* 1–88.

**van de Geld, P. A., Oosterveld, P., van Waas, M. A., & Kuijpers-Jaqtman, A. M.** (2007). Digital videographic measurement of tooth display and lip position in smiling and speech: Reliability and clinical application. *American Journal of Orthodontics and Dentofacial Orthopedics, 131,* e1–e8.

**Vander Werff, K., Prieve, B., & Georgantas, L.** (2007). Test–retest reliability of wideband reflectance measures in infants under screening and diagnostic test conditions. *Ear and Hearing, 28,* 669–681.

**Vanhuyse, V. J., Creten, W. L., & Van Camp, K. J.** (1975). On the W-notching of tympanograms. *Scandinavian Audiology, 4,* 45–50.

**Werner, L. A., Levi, E. C., & Keefe, D. H.** (2010). Ear-canal wideband transfer functions of adults and two- to nine-month-old infants. *Ear and Hearing, 31,* 1–12.

**Appendix** (p. 1 of 2)

A Concise Overview of Generalizability Theory (GT)

The conceptual basis of GT requires a detailed understanding of repeated measures analysis of variance (ANOVA); tutorial and computational methods are available to assist clinicians or researchers in this regard (see Di Nocera, Ferlazzo, & Borghi, 2001; Mushquash & O'Connor, 2006). However, we caution that there is no simple "cookbook" approach to data analysis with GT. This is due in part to the many design features that are possible within a given experimental framework. Thus, as would be the case for any experimental design using ANOVA, the approach taken will depend on the complexity of the experiment and the statistical model being used.

In Figure A1, two hypothetical cases involving four test sessions in three subjects are shown in diagrammatic form. The figure illustrates a case in which the relative ranking of each subject's test scores is consistent across test sessions (Panel A). In this case, the main effect of subjects is large relative to the interaction between subjects and test sessions. In a second case, the ranking of the subjects' test scores varies considerably across test sessions (Panel B). This results in a large interaction between subjects and test sessions relative to the main effect of subjects. The generalizability coefficient is a ratio of the main effect of subjects to the sum of that main effect and the interaction between subjects and test sessions. Thus, this ratio would be much larger in the first case than in the second.

The metric used in GT is expressed as the proportion of the total variance due to subjects that is common to the testing occasions of interest. For a single measure determined on two testing occasions, this can be computed as Pearson's *r*; for *k* testing occasions, it is computed as

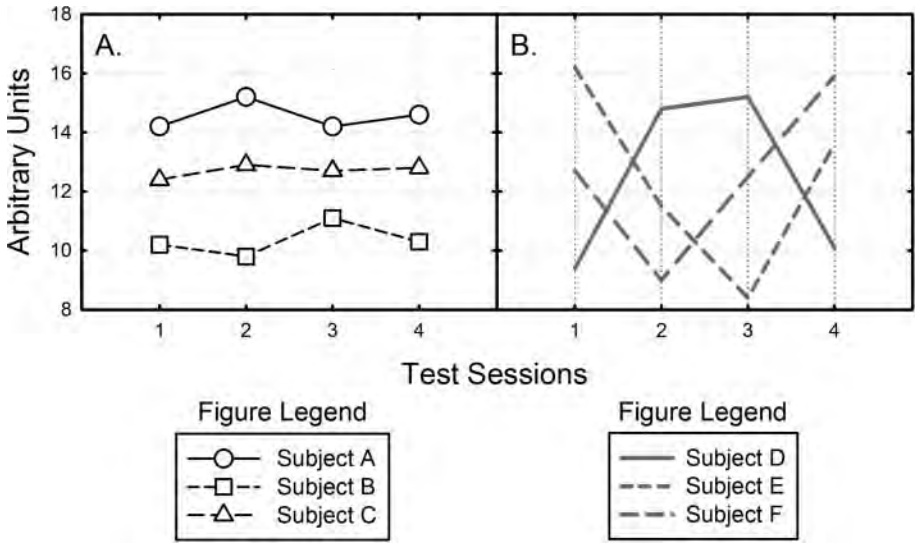
$$\rho^2 = \sigma^2_{subj}/(\sigma^2_{subj} + \sigma^2_{err}), \quad (A1)$$

where $\sigma^2_{subj}$ is the variance due to the main effects of subjects and $\sigma^2_{err}$ is the variance due to error (i.e., the nonadditive or inconsistent effect of subjects across testing occasions).

As a relevant example, we present a "thought experiment" whereby the reliability of middle-ear power reflectance is assessed at 1500 Hz for the right ear on two occasions. Here, our sample consists of 56 adults without a history of middle-ear disease.

Results from the ANOVA are depicted in Table A1. Degrees of freedom, sum of squares, mean square, and expected mean square can be obtained from many statistical programs that are commercially available in the marketplace (e.g., SAS, SPSS, Statistica, etc.). Statistical programs such as these report *F* values and probability estimates (*p* values) associated with the effects of time of testing and their significance. The *F* for the effect of time is simply the ratio obtained by dividing the value in Row 2 by

**Figure A1.** Examples of tests with differing generalizability coefficients. In both plots, each of three subjects' scores on four test sessions is represented by a line. The main effect of subjects represents the variance in the average difference between subjects. This is large when the profiles are parallel. The interaction between subjects and sessions represents the extent to which the ordering of subjects varies across sessions; it is large when the profiles are nonparallel. In Panel A, one can see that the three subjects performed relatively consistently across the four test sessions. As a result, the main effect of subjects is large relative to the interaction between subjects and test sessions. In Panel B, one can see that the three subjects performed inconsistently across the four test sessions. As a result, the main effect of subjects is small relative to the interaction between subjects and test sessions.



**Appendix** (p. 2 of 2)

A Concise Overview of Generalizability Theory (GT)

**Table A1.** Summary of example "thought experiment" of power reflectance at 1500 Hz in the right ear for 56 subjects tested on two occasions.

| Source | *df* | SS | MS | EMS | Subjects pooled | Subjects single test |
|---|---|---|---|---|---|---|
| Time | 1 | 13.99 | 13.99 | | | |
| Subjects | 55 | 14,794.53 | 268.99 | $\sigma^2_{err} + k\,\sigma^2_{subj}$ | 0.917 | 0.847 |
| Time × Subjects | 55 | 1,224.78 | 22.27 | $\sigma^2_{err}$ | | |

*Note.* SS = sum of squares; MS = mean square; EMS = expected mean square.

that in Row 1 for the mean-square value. In this example, the $p$ value is not significant ($p > .05$; result not shown). The expected mean square (*EMS*) for the effects of subjects is

$$EMS_{subj} = k\sigma_p^2 + \sigma_{err}^2, \tag{A2}$$

after Crocker and Algina (1986). The mean square (*MS*) for subjects represents the variance summed over all testing occasions and associated error. The *EMS* for the error is $MS_{time \times subj}$ and represents the nonadditive effect of subjects over time (i.e., the effect of test sessions), often computed as the residual in this design:

$$\sigma_{subj}^2 = (MS_{subj} - MS_{err})/k. \tag{A3}$$

A pooled estimate of the proportion of the variance in subject scores that is consistent across time (additive) can be obtained by the following equation:

$$\rho_{pooled}^2 = (MS_{subj} - MS_{err})/(MS_{subj} - MS_{err}) + MS_{err}. \tag{A4}$$

This represents the reliability of a composite of the test scores summed across time and is reported under "Subjects pooled" in Table A1. Because one is usually interested in the reliability of single tests, this is computed as

$$\rho^2 = [(MS_{subj} - MS_{err})/k]/[(MS_{subj} - MS_{err})/k + MS_{err}], \tag{A5}$$

and takes into account the estimate of single test variance from Equation A3 and is reported under "Subjects single test" in Table A1.

More complex models that include additional facets that interact with subjects can also be constructed. For example, if separate measurements were taken for each ear on several occasions, then an Ear × Subjects interaction could be computed. In this case, the model would be

$$\rho^2 = [(MS_{ear \times subj} - MS_{err})/ek]/[(MS_{ear \times subj} - MS_{err})/ek + MS_{err}], \tag{A6}$$

where $e$ represents the number of ears and $k$ represents the number of test sessions. The $MS_{err}$ is now the value of $MS_{time \times ear \times subj}$. Thus, interactions between subjects and various measures repeated across subjects can be generated by substituting these interaction terms for the main effect terms in Equation A5. Conceptually, in a model including subjects, ears, and occasions, the generalizability coefficient associated with the main effects of subjects ($MS_{subj}$) represents the reliability of a score averaged across ears, whereas the generalizability coefficient associated with the Ear × Subjects interaction represents the reliability of a score as the difference between the ears. In fact, a wide variety of models can be generated following the logic of the general linear model (see Laenen et al., 2006).

Although statistical packages such as SAS, SPSS, or Statistica do not offer explicit tools for computing generalizability coefficients, the individual *MS* values are provided in the standard ANOVA summary table for a model including time as a repeated measure (i.e., in within-subject designs). Thus, generalizability coefficients can be readily computed with just a few simple operations. To aid in these computations, Mushquash and O'Connor (2006) provided a guide for users of SAS, SPSS, or MATLAB.

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Research paper

# Left hemisphere fractional anisotropy increase in noise-induced tinnitus: A diffusion tensor imaging (DTI) study of white matter tracts in the brain

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ABSTRACT

Diffusion tensor imaging (DTI) is a contemporary neuroimaging modality used to study connectivity patterns and microstructure of white matter tracts in the brain. The use of DTI in the study of tinnitus is a relatively unexplored methodology with no studies focusing specifically on tinnitus induced by noise exposure. In this investigation, participants were two groups of adults matched for etiology, age, and degree of peripheral hearing loss, but differed by the presence or absence (+/−) of tinnitus. It is assumed that matching individuals on the basis of peripheral hearing loss, allows for differentiating changes in white matter microstructure due to hearing loss from changes due to the effects of chronic tinnitus. Alterations in white matter tracts, using the fractional anisotropy (FA) metric, which measures directional diffusion of water, were quantified using tract-based spatial statistics (TBSS) with additional details provided by *in vivo* probabilistic tractography. Our results indicate that 10 voxel clusters differentiated the two groups, including 9 with higher FA in the group with tinnitus. A decrease in FA was found for a single cluster in the group with tinnitus. However, seven of the 9 clusters with higher FA were in left hemisphere thalamic, frontal, and parietal white matter. These foci were localized to the anterior thalamic radiations and the inferior and superior longitudinal fasciculi. The two right-sided clusters with increased FA were located in the inferior fronto-occipital fasciculus and superior longitudinal fasciculus. The only decrease in FA for the tinnitus-positive group was found in the superior longitudinal fasciculus of the left parietal lobe.



## 1. Introduction

Noise-induced hearing loss (NIHL) is a ubiquitous phenomenon observed in modern society (Clark and Bohne, 1999). It is also a prominent concern for individuals serving in the military (Humes et al., 2006) and represents a known risk factor for developing tinnitus (e.g., Dong et al., 2010; Henderson et al., 2011; Yankaskas, 2012). Epidemiological studies indicate that as hearing thresholds exceed the mild range of severity in the better ear at 4.0 kHz, the odds ratio for expressing moderate to severely annoying tinnitus increases from 5 to 27 (Coles, 2000). Other studies support this observation by documenting that over 83% of individuals with NIHL have tinnitus (Mazurek et al., 2010). While mechanisms underlying tinnitus expression are not completely known, available evidence suggests that temporary or permanent damage to sensory cells in the inner ear is a known trigger of this phenomenon, resulting in temporary or permanent elevations in auditory thresholds, physiological changes in auditory nerve discharge properties (e.g., Liberman and Kiang, 1978; Henderson et al., 2011; Kujawa and Liberman, 2009) and secondary anatomical and physiological effects in the central auditory nervous system (e.g., Morest et al., 1998; Salvi et al., 2000; Syka, 2002; Schreiner and Cheung, 2004). These observations suggest that reduced afferent drive from the periphery leads to an imbalance between inhibitory and excitatory input to auditory neurons at various levels in central auditory pathways (e.g., Middleton et al., 2011; Wang et al., 2011; Brozoski et al., 2012; Browne et al., 2012; Godfrey et al., 2012). This altered

*Abbreviations:* ATR, anterior thalamic radiations; dB, decibels; DTI, diffusion tensor imaging; FA, fractional anisotropy; FMRIB, functional magnetic resonance imaging of the brain; FSL, functional software library; GABA, gamma-aminobutyric acid; ICBM, International Consortium of Brain Imaging; MRI, magnetic resonance imaging; NIHL, noise induced hearing loss; ROI, region-of-interest; SLF, superior longitudinal fasciculus; TBSS, tract based spatial statistics; TE, echo time; TR, repetition time

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pattern of activity is thought to destabilize circuits in the brainstem and cortex resulting in plastic readjustments attempting to compensate for this disparity (e.g., Eggermont and Roberts, 2004; Roberts et al., 2010). Either alone or in combination, neuronal hyperactivity, bursting discharges, and increased cortical or brainstem/thalamic neural synchrony are thought to be the neurophysiological substrates that result in the perception of tinnitus (e.g., Dong et al., 2010; Henderson et al., 2011; Kaltenbach, 2011; Brozoski et al., 2012). Interestingly, while excessive noise exposure can produce anatomical, physiological, and biochemical changes in peripheral and central auditory pathways, not all individuals with NIHL or other otopathologies develop tinnitus (e.g., Lockwood et al., 2002). Indeed, the precise reasons for this discrepancy remain unknown.

To better understand the neurobiology of noise-induced tinnitus and evaluate its relationship, if any, to white matter changes in the brain, diffusion-tensor imaging (DTI) was used as a platform for discovery. Diffusion-tensor imaging represents a neuroimaging modality that can provide insight into plastic/reactive changes in white matter microstructure and connectivity associated with tinnitus that cannot be detected by conventional magnetic resonance imaging (MRI). Specifically, this imaging modality measures the displacement of water molecules (diffusion) within white matter tracts, providing information on the microstructure of cerebral white matter and thus serves as a biomarker of tissue integrity (e.g., Ling et al., 2012). For each voxel, DTI estimates diffusion in three orthogonal axes (eigenvectors) of an ellipsoid, defining the principal (major), intermediate, and minor axes. The most commonly used metric to quantify the relationship between eigenvalues is fractional anisotropy (FA), a normalized scalar that represents the fraction of the diffusion tensor which is anisotropic. Herein, we focus on FA, because it reveals information regarding fiber integrity and network reorganization, i.e., activity dependent neuroplasticity (Yu et al., 2007; Scholz et al., 2009; Steele et al., 2013).

The FA metric ranges between 0 and 1, where 0 represents perfectly "isotropic" diffusion, such as is found in the cerebrospinal fluid where diffusion is equivalent in all directions, and where 1 is the extrema for "anisotropic" diffusion, indicating maximum difference between directional components, such as is found in coherent white matter tracts which consist of long tubes.

Prior use of DTI in tinnitus research is limited to a small number of studies with mixed results (e.g., Lee et al., 2007; Crippa et al., 2010; Husain et al., 2011; Aldhafeeri et al., 2012). These studies are reviewed below.

Lee et al. (2007) used DTI to study adults with mild-to-severe hearing loss and tinnitus ($n = 28$; 11 female, 17 males; age range 22–70 years) and compared results to a group of "normal hearing" younger controls ($n = 12$; 6 males, 6 females; age range 22–34 years). While they indicated that 12 had left-sided tinnitus for a duration of 1–8 years (mean duration 3 years), details regarding etiology were not provided. In comparison to the control group, Lee et al. (2007) found significant *reductions* in FA in the left frontal and right parietal arcuate fasciculus. However, because the control group was not matched for hearing loss or age, it would be difficult to differentiate white matter changes due to tinnitus from changes due to hearing loss or age effects. Thus, these findings are indeterminate with respect to tinnitus-related dysfunction.

Crippa et al. (2010) used region-of-interest (ROI) analysis and probabilistic tractography as methods to study DTI changes in tinnitus in 15 healthy subjects and 10 subjects with tinnitus which were matched for age but not explicitly for hearing thresholds. In theory, probabilistic tractography is of interest because it allows investigators to resolve voxels with multiple fiber directions that cross in a voxel. This ROI analysis scheme helps to isolate white matter fiber connections in classical (inferior colliculus to auditory cortex) and non-classical (auditory cortex to amygdala) auditory pathways. Their approach was partially successful in that the tinnitus group showed a right lateralization of increased FA values which they describe as "an increased patency of the white matter tracts between the auditory cortex and the amygdala in tinnitus patients as compared to healthy controls, p. 16." This difference in the non-classical pathway, however, may be a consequence of chronic exposure to an unpleasant or distressing perception rather than a clue to the generation mechanism of the tinnitus perception.

Husain et al. (2011) were unable to detect differences in FA in adults with tinnitus ($n = 8$) in comparison to age and hearing loss matched controls without tinnitus ($n = 7$) and normal hearing controls ($n = 11$). Possible reasons for their negative results include: a small sample size, heterogeneity of etiology of hearing loss and/or tinnitus, and the fact that the average severity rating of tinnitus in their sample was in the "mild" range.

Aldhafeeri et al. (2012) studied cortical and white matter FA and diffusivity in healthy volunteers with no tinnitus and normal hearing ($n = 14$; 9 males, 5 females; age range 30–60 years, mean age 46.5 years) and participants with tinnitus and hearing loss, described as no worse than 40 dB HL at 2.0 kHz and 60 dB HL at 4.0 kHz ($n = 14$), 8 males, 6 females, 30–60 years of age, mean age 49.5 years. Whereas cortical thickness was found to negatively correlate with hearing thresholds, in comparison to controls, the participants with tinnitus and hearing loss showed reduced white matter FA values in the right prefrontal cortex, right auditory cortex, and corpus callosum. However, this study was also limited by the fact that comparisons of FA were made between a tinnitus positive group *with* hearing loss to a control group *without* hearing loss.

In summary, out of 4 studies using DTI to study tinnitus, 1 had negative results (Husain et al.), 2 had inadequate control groups (Lee et al., 2007; Aldhafferi et al., 2012) and 1 showed positive results (Crippa et al., 2010) but their novel quantification methodology requires further development in order to be successfully applied to all participants. Nevertheless, both whole brain (voxel-wise) and ROI DTI analyses have proven successful in identifying brainstem and cortical pathway anomalies in tinnitus positive groups. While there are both strengths and weaknesses to each type of analysis methodology (e.g., Snook et al., 2007), combining both procedures may be the most comprehensive approach to be applied in future endeavors in this area.

Therefore, to enhance understanding in this area, improve the specificity of results and to help distinguish white matter changes due to pathologic plasticity associated with noise-induced tinnitus from white matter changes due to hearing loss, we studied two groups of individuals, using whole brain and ROI approaches, matched for NIHL but differing in presence or absence of tinnitus.

## 2. Materials

### 2.1. Subjects

Two groups of adults with a common history of occupational, recreational, or military noise exposure, *with* and *without* tinnitus, matched for degree/severity of hearing loss and age were evaluated. Participants were recruited from newspaper ads, referrals from medical and/or allied health professionals in the greater Detroit metropolitan area (i.e., otolaryngologists, neurologists, audiologists) and by word-of-mouth. Group 1 consisted of adults with NIHL *without* tinnitus ($n = 13$, mean age 58 years, range 22–88 years); Group 2 consisted of adults with NIHL *with* tinnitus ($n = 13$, mean age 54 years, range 28–80 years). The selection criteria utilized was based on the assumption that homogeneous groups of individuals with similar etiologies will improve biomarker

specificity and that matching groups with respect to as many relevant factors as possible, will be beneficial to enhance our understanding of this condition.

#### 2.1.1. Inclusion criteria

All participants with tinnitus were required to have chronic/constant tinnitus over at least the prior 6-month period and score in the moderate or higher range of severity on the Tinnitus Handicap Inventory (score; ≥35) (Newman et al., 1996; also see Newman and Sandridge, 2004 for severity score criteria).

#### 2.1.2. Exclusion criteria

Documented history of blast exposure, retro-cochlear or neurologic disease (e.g., auditory nerve and/or skull-base tumors, brain tumors, strokes, demyelinating disease, etc.), active use of GABAergic agonist medications, and/or other pharmaceutical compounds used to treat depressive illness.

This study was approved by the Human Investigation Committee of Wayne State University and signed informed consent was obtained from each individual prior to data collection.

### 2.2. DTI image acquisition

Magnetic resonance imaging data were collected at the MR Research Facility of Wayne State University located at Harper Hospital of the Detroit Medical Center. For data acquisition, a 3 T Siemens MAGNETOM Verio scanner employing a 32-channel head coil with diffusion-sensitizing gradients applied in 20 non-collinear directions was utilized. Diffusion tensor imaging data was acquired with a diffusion-weighted single shot spin-echo (i.e., echo-planar imaging sequence), where: TR = 7400 ms, TE = 106 s, flip angle = 90°, in-plane resolution = 2 × 2 $mm^2$, slice thickness = 3 mm and number of excitations = 2. Twenty-one volumes were collected including 1 without diffusion weighting ($b = 0$ s/$mm^2$) and 20 with diffusion weighting ($b = 1000$ s/$mm^2$).

## 3. Methods

### 3.1. Audiologic testing

Audiometric testing was conducted in a commercial sound booth (Acoustic Systems, Austin, Texas; Model RE-144) using a clinical audiometer (Grason-Stradler, model 61) with standard earphones (Telephonics, TDH-50P) enclosed in supra-aural ear cushions (MX-41/AR). Pure-tone air-conduction audiometry was performed at octave frequencies from 0.25 through 8.0 kHz and also at one half-octave frequency (3.0 kHz) bilaterally. Bone conduction testing used a standard oscillator (Radioear B-71) and headband; bone-conduction thresholds were assessed at octave frequencies from 0.25 through 4.0 kHz. Additionally, if any of the participants had audiological testing performed within 3 months of enrollment in the study by an ASHA certified, state licensed, or Veterans Administration audiologist, then, this type of testing was not repeated.

### 3.2. DTI image processing

DTI Studio (Jiang et al., 2006) (https://www.dtistudio.org/) was used. All individual images were visually inspected to discard slices with motion artifacts, after which the remaining images were added for each slice. The pixel intensities of the multiple diffusion-weighted images were then fitted to obtain the six elements of the symmetric diffusion tensor. The diffusion tensors at each pixel were diagonalized to obtain pixel eigenvalues and eigenvectors. Fractional anisotropy maps of the diffusion tensor were obtained for additional analyses.

### 3.3. Voxel-wise analysis

For whole brain voxel-wise analysis of FA, tract-based spatial statistics (TBSS) (Smith et al., 2006) was used. This is part of the Oxford Center for Functional Magnetic Imaging of the Brain (FMRIB) Software Library (FSL), which is a compendium of computer programs containing image analysis and statistical procedures for evaluating functional, structural, and diffusion MRI data of the brain (Smith et al., 2004; also see http://www.fmrib.ox.ac.uk/fsl/). Fractional anisotropy maps from each individual were co-registered using the nonlinear image registration toolkit (Rueckert et al., 1999) (www.doc.ic.ac.uk/~dr/software) to a single subject. After image registration, FA maps were averaged to produce a group mean image. A skeletonization algorithm was applied to the group mean FA image to define a group template of the lines of maximum FA, corresponding to centers of white matter tracts. Fractional anisotropy values for each participant were then projected onto the group skeleton template by searching along perpendiculars from the skeleton to find local maxima. Voxel-wise analyses of FA across the group of subjects were performed only on data projected onto the skeleton template (which is recruited from the nearest tract center in each subject's image). Comparison of white matter microstructural differences between the two groups which differed by presence of tinnitus used a voxel-wise permutation-inference analysis (5000 permutations) which is included in the TBSS protocol. Other parameters used were an FA minimum threshold of 0.3 to avoid partial volume effects with gray matter and a voxel-wise lower threshold of $t = 3$ for cluster analysis to detect regional group differences.

### 3.4. Probabilistic tractography

Probabilistic tractography is performed to quantify connectivity between brain regions. Our approach in the current study was to not restrict the number of brain regions to classical auditory areas but to consider the entire volume of white matter, understanding that the tinnitus generator mechanism(s) and post-perceptual processing, including long-term plastic changes, may be complex and involve classical as well as non-classical neural networks. Therefore, probabilistic tractography can serve three important functions: 1) to more fully delineate the fiber tract containing a "hotspot," i.e., a region that is shown to differentiate tinnitus from non-tinnitus; 2) to track the gray-matter regions sourcing the fibers; and 3) to identify any group differences in fiber tractography that extend beyond ROI differences.

The BEDPOST tool (Behrens et al., 2003), which runs a Markov Chain Monte Carlo sampler, was used for building distributions of parameters describing the diffusion direction in each voxel. Probabilistic tractography was accomplished using the FMRIB Diffusion Toolkit (Behrens et al., 2003). For each voxel in an ROI seed mask, 5000 samples were taken from the distribution. Probabilistic tractography outputs a connectivity map indicating the voxel-wise probability of reaching a given voxel starting from a user-defined ROI, and is defined as the percentage of samples leaving from the starting ROI that pass through that voxel. Probabilistic tractography (FSL), using default parameters, was run using each TBSS-obtained significant cluster (see 3.3) as a seed mask for each of the 26 subjects. Tractography allowed for determination of paths for fiber bundles passing through a given seed mask. This allowed for determination of gray matter "origin" and "termination" of the fiber paths. The interactive Brodmann's atlas contained in MRIcro (Tzouio-Mazoyer et al., 2002) was used to determine these gray

matter sites. Two group-mean tractography maps were generated for all 9 significant clusters obtained in the TBSS voxel-wise step.

## 4. Results

### 4.1. Audiograms

Fig. 1 shows audiograms of the tinnitus and non-tinnitus groups, matched for degree of hearing loss. On average, pure tone hearing sensitivity was in the normal range from 0.25 to 2.0 kHz and increased in severity from 3.0 to 8.0 kHz. Loss of pure tone hearing sensitivity was bilateral, generally symmetric, and ranged from mild-to-severe. In all cases, hearing loss was sensorineural in nature. Top panels (A and B) show pure-tone audiograms for individuals with NIHL *without* tinnitus; bottom panels (C and D) show pure-tone audiograms for individuals *with* NIHL with tinnitus. The degree and configuration of hearing loss did not differentiate those with and without tinnitus. To reiterate, we assumed that by controlling for degree of hearing loss, differences in white matter FA between groups could be attributable to tinnitus-related effects.

### 4.2. ROI analysis

Global white matter FA did not differ significantly between groups ($t = 2.06$, $p > 0.05$). However, voxel-wise analysis revealed 9 regions which showed *increased* FA for the tinnitus positive (+) group ($p < 0.0001$) (see Table 1). The majority (7 of 9) were left-sided, including 5 regions localized to left anterior thalamic radiations (ATR), 1 in the superior longitudinal fasciculus (SLF), and 1 in the inferior longitudinal fasciculus. The 2 right-sided regions were localized to the inferior fronto-occipital fasciculus (inferior fronto-occipital fasciculus) and SLF. Fig. 2 gives the distribution characteristics (central tendency measures, means/medians; and dispersion, percentiles) of FAs by group for each of the 9 significant clusters using box and whisker plots.

### 4.3. Tractography

Tractography revealed the paths for the 9 cluster seed masks. Of the 9 fiber tract paths, 6 were in either the ATR (4) or SLF (2). The others tracts were in the superior corona radiata, inferior longitudinal fasciculus and the inferior fronto-occipital fasciculus. Table 1 gives the gray matter origin and termination for each of the 9 fiber tracts. These gray and white matter structures comprised a broad network of mostly left hemisphere brain regions which included: left inferior frontal gyrus (Brodmann's area 44), premotor cortex (area 6), supramarginal gyrus (area 40), prefrontal cortex (areas 10, 11 and 46), corticospinal tract, and anterior thalamus. Right sided regions included: inferior and middle temporal gyri (areas 20, 21), inferior frontal gyrus (area 47), motor cortex (areas 4 and 6), orbitofrontal cortex (area 11), and extrastriate visual cortex (area 18).

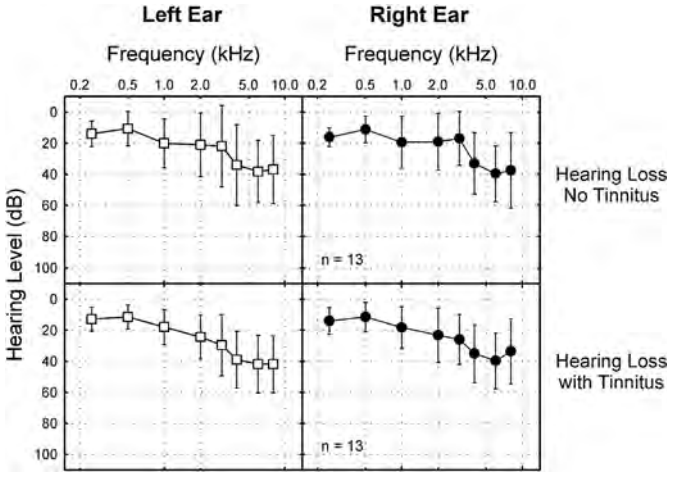


**Fig. 1.** Audiograms. Top panels represent audiograms (mean ±1SD) for left (unfilled squares) and right ears (filled circles) for individuals without tinnitus; bottom panels represent audiograms (mean ±1SD) for left (unfilled squares) and right ears (filled circles) for individuals with tinnitus.

Not only did the clusters discriminate the two groups of subjects, the *in vivo* probabilistic tractography generated from these clusters also revealed white matter tract differences between the groups. Fig. 3 gives the paired group-averaged tractography results for each of the 9 clusters (labeled A–I). Seven of the 9 clusters with higher FA for the tinnitus group also revealed greater continuity of connectivity strength which can be appreciated in the tractography results.

## 5. Discussion

The major findings in this study were the presence of clusters of *increased* FA in the white matter in a group of individuals *with* moderate-to-severe noise-induced tinnitus compared with a similar group of individuals matched for etiology, age and degree of hearing loss but *without* tinnitus. These white matter foci localized predominantly to the ATR, SLF and inferior longitudinal fasciculus of the left hemisphere. These neuroanatomical findings and their connectivity patterns suggest a role of the ATR, SLF and inferior longitudinal fasciculus in noise-induced tinnitus.

Anterior thalamic radiations project to the frontal lobe (areas 10, 46) and anterior cingulate cortices (Zhou et al., 2001; Mamah et al., 2010). The SLF, consisting of three components (I, II, III), projects from the fronto-temporal and fronto-parietal regions in a bidirectional manner and the inferior longitudinal fasciculus interconnects occipital with ipsilateral temporal lobes (e.g., Catani et al., 2002; Makris et al., 2005).

While these specific white-matter tracts can be considered default pathways when viewed in the context of cortical connectivity of the normal brain, the increased FA for these fiber pathways indicates neuroplastic changes in these pathways as a result of increased connectivity between these neural loci.

### 5.1. MR spectroscopy studies

Recent neurobiochemical data in rats with behavioral evidence of tinnitus induced by noise exposure is illuminating with respect to understanding our results. Using high-field proton ($^1$H) magnetic resonance spectroscopy, Brozoski et al. (2012) showed a down-regulation of the neurotransmitter gamma-aminobutyric acid (GABA) at the level of the thalamus and an up-regulation of glutamate in the dorsal cochlear nucleus and in primary auditory cortex. Accordingly, these "bidirectional" alterations in glutamate and GABA may reflect large-scale changes in excitatory and inhibitory neurotransmission, respectively, that may underlie the generation and then maintenance of noise-induced tinnitus (Brozoski et al., 2012; also see Kaltenbach, 2011 for a review). The increased FA in the thalamic radiations found in our study is likely the downstream consequence of these local changes in synaptic activity which over time lead to increases or decreases in connectivity (neuroplasticity) in response to acoustic trauma (e.g., Mulders and Robertson, 2011; Mulders et al., 2011).

### 5.2. Increased FA in neurological disorders

While the axonal morphological changes underlying increased FA in noise-induced tinnitus remain unknown, increases in FA have

**Table 1**
Results by cluster (A–I) of TBSS analysis of tinnitus (+) vs. tinnitus (−) groups. All clusters have increased FA for tinnitus (+) group.

| Cluster index | *T*-stat ($t > 3$) local max. | *P*-value local max. | Cluster size, in voxels | Mean *P*-value of cluster | Local max. | | | JHU ICBM-DTI-81 labels | *L*/*R* | Origin | Termination | JHU-white matter tractography |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *X* | *Y* | *Z* | | | | | |
| A | 5.54 | 1.50E-08 | 21 | 3.45E-04 | 138 | 121 | 93 | Superior longitudinal fasciculus | *L* | 40 | 6, 44, 46 | Superior longitudinal fasciculus III |
| B | 4.93 | 4.10E-07 | 10 | 5.57E-04 | 121 | 169 | 81 | Anterior corona radiata | *L* | Ant thalamus | 10, 46 | Anterior thalamic radiation |
| C | 4.75 | 1.00E-06 | 18 | 5.17E-04 | 107 | 123 | 122 | Superior corona radiata | *L* | 6 | CST | Superior corona radiata |
| D | 4.62 | 1.90E-06 | 23 | 4.86E-04 | 110 | 64 | 107 | SLF/SCR | *L* | Temporal | Occipital | Inferior longitudinal fasciculus |
| E | 4.35 | 6.70E-06 | 13 | 2.97E-04 | 67 | 68 | 106 | SLF/SCR | *R* | 11, 47 | 18 | Inferior fronto-occipital fasciculus |
| F | 4.2 | 1.30E-05 | 12 | 4.73E-04 | 112 | 136 | 89 | Ant. limb of internal capsule | *L* | Ant thalamus | 10, 46 | Anterior thalamic radiation |
| G | 3.9 | 4.70E-05 | 11 | 5.57E-04 | 106 | 128 | 82 | Ant. limb of internal capsule | *L* | Ant thalamus | 10, 46 | Anterior thalamic radiation |
| H | 3.88 | 5.10E-05 | 13 | 5.08E-04 | 56 | 117 | 96 | Superior longitudinal fasciculus | *R* | 20, 21 | 6, 4 | Superior longitudinal fasciculus II |
| I | 3.79 | 7.40E-05 | 15 | 5.59E-04 | 105 | 132 | 81 | Ant. limb of internal capsule | *L* | Ant thalamus | 10, 46 | Anterior thalamic radiation |

Local max. = maximum *t*-value of the local maximum of the cluster obtained from TBSS; *P*-value local max. = corresponding *P*-value of the local maximum; Local max. *x*, *y*, *z* = coordinate in ICBM space of local maximum; Johns Hopkins University ICBM-DTI-81 = anatomical label of a cluster, derived from a DTI atlas which averaged FA images from 81 normal volunteers; Origin and termination = tractography-derived gray matter destinations; Johns Hopkins University White matter tractography = atlas-derived tract name (Johns Hopkins University ICBM-81 atlas); SLF = superior longitudinal fasciculus; SCR = superior corona radiata.

been related to increased myelination, decreased axonal diameter, decreased axonal branching and increased packing density of white matter fibers (Beaulieu, 2002). In addition to results found in the current study, increased and decreased FA values have been reported in other disorders of the brain, including: Williams (aka, Williams-Beuren) syndrome (Hoeft et al., 2007; Arlinghaus et al., 2011; Haas and Reiss, 2012; Haas et al., 2012), juvenile myoclonic epilepsy (Keller et al., 2011), cryptogenic temporal-lobe epilepsy (Rugg-Gunn et al., 2001), recurrent psychomotor seizures (Gerdes et al., 2012), Huntington's disease (Klöppel et al., 2008; Douaud et al., 2009), pantothenate kinase-associated neurodegeneration formerly known as Hallervorden-Spatz disease (Awasthi et al., 2010), brain tumors (e.g., gliomas) associated with hemorrhage (Harris et al., 2006), attention deficit hyperactivity disorder (Li et al., 2010), developmental and adult-onset stuttering (Chang et al., 2010), bipolar disorder (Versace et al., 2008), and mild traumatic brain injury (Gattu et al., 2012; Ling et al., 2012). The plethora of neurologic and behavioral disorders associated with FA abnormalities suggests that FA is likely a local marker of aberrant or increased neural connectivity. Whether increased FA/connectivity

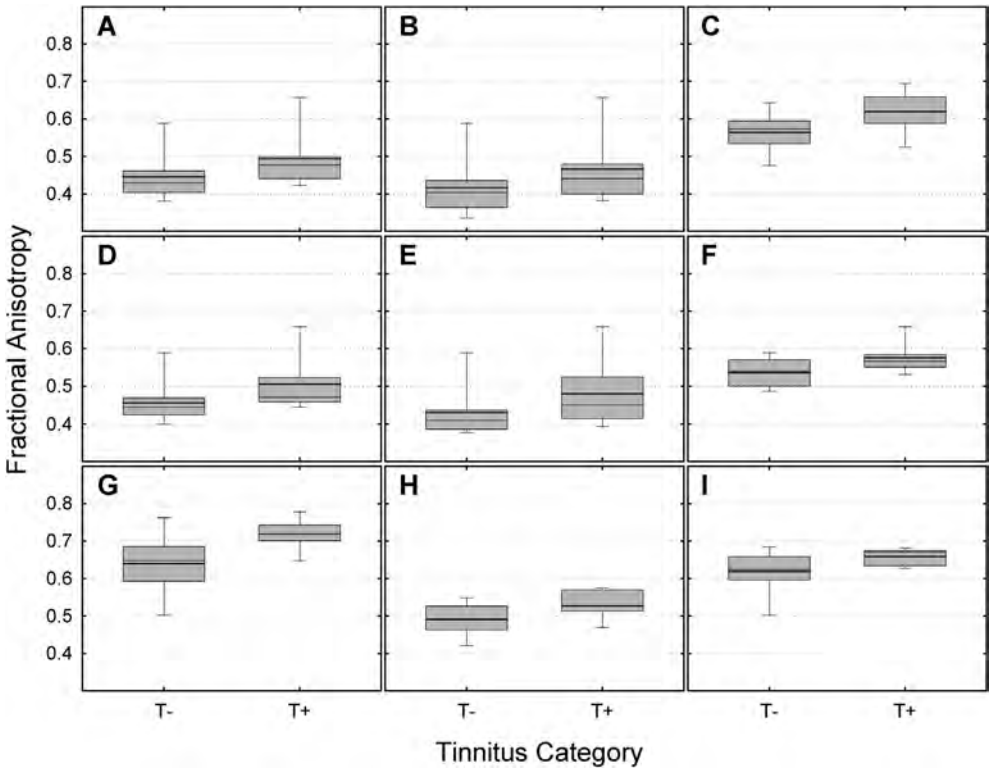


**Fig. 2.** Box and whisker plots of mean FA for clusters A–I for tinnitus (−) group and tinnitus (+) group. Each box shows means (thin line with the box), medians (thick line within the box), and 5th and 95th percentiles (top and bottom error bars) giving the range of FA values for subjects in each group. *Y*-axis is FA values; T− is Tinnitus negative; T+ is Tinnitus positive.

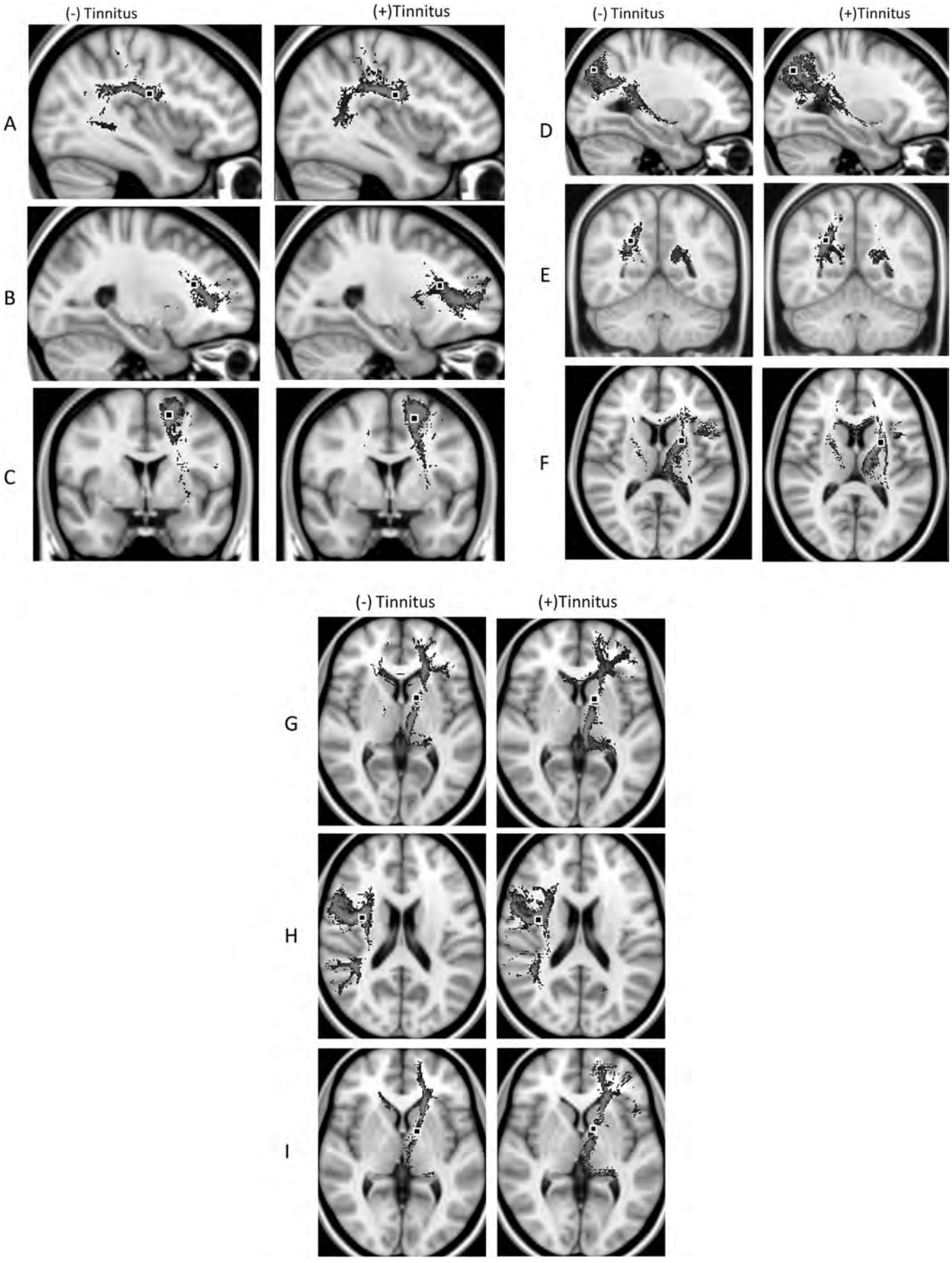


**Fig. 3.** Group-averaged tractography results for each cluster. See text 3.4 for explanation of how tractography was performed. The white square shows the cluster which was used as the seed mask for tractography. The slice selected for display of tractography results was chosen to show the longest continuous path length in either group. Grey scale: grey is highest probability of passage; black is lowest probability of passage. The spatial location of the left and right sides of the MRI corresponds to standard radiological convention.

is causally related to the behavioral/neurological abnormalities observed in the above disorders or is a secondary, compensatory alteration in neural connectivity, may vary by particular disorder. In the case of noise-induced tinnitus, it is likely that central nervous system alterations in FA reflect a new steady state of functional and anatomical connectivity induced by peripheral end organ-induced damage/deafferentation.

What makes Williams syndrome unique in this regard is the fact that auditory abnormalities are a prominent feature in conjunction with other medical issues.[1] In addition to increased FA values in white matter tracks in Williams syndrome reported by Hoeft et al. (2007), well-described auditory anomalies such as hyperacusis (Levitin et al., 2005; Attias et al., 2008; Matsumoto et al., 2011; Elsabbagh et al., 2011), auditory allodynia (Levitan and Bellugi, 1998; Levitin et al., 2003, 2005; Miani et al., 2001; Nigam and Samuel, 1994), dysfunction of the olivocochlear efferent system (Attias et al., 2008) and structural anomalies of auditory cortical structures have been reported. These cortical anomalies include reduced volume and altered sulcal morphology of the Sylvian fissure, atypical cytoarchitecture in Heschl's gyrus, and increased volume of the superior temporal gyrus (Eckert et al., 2006; Holinger et al., 2005; Reiss et al., 2004). Interestingly, the auditory system anomalies are similar to those observed in individuals with tinnitus (Mahoney et al., 2011; Boyen et al., 2013; Schecklmann et al., 2013).

Gattu et al. (2012) have shown a distinct pattern of evolution of FA over 72 h, with an early increase within 4 h followed by a decrease at 24 h, using an impact-acceleration rodent model where DTI was compared with histopathologic markers of axonal injury.

Lastly, in a DTI study of idiopathic developmental and adult-onset stuttering, using an ROI analysis, Chang et al. (2010) found increased FA in the developmental stuttering group localized to the Rolandic operculum of the right hemisphere, with a similar trend found in the adult cases. The authors state that the increased FA may result from "compensatory neuroplasticity in response to impairments in the left hemisphere, p. 8."

### 5.3. fMRI studies of tinnitus

A related perspective is the recent functional magnetic resonance imaging (*f*MRI) study of Gu et al. (2010) in individuals with tinnitus. Using binaurally presented white noise stimuli at multiple levels (50, 70, 80 dB SPL), Gu and colleagues studied individuals with normal hearing with and without tinnitus and evaluated the relationship of these groups to measures of sound level tolerance (hyperacusis) based on questionnaire data, loudness discomfort levels, and perceived loudness based on a 7 point numerical rating scale. The general *f*MRI activation patterns in regions-of-interest within midbrain (inferior colliculus) and thalamus (medial geniculate body) showed a dependence on percent signal change on sound level tolerance but not on tinnitus. In contrast, only primary auditory cortex and core regions (anterior lateral Heschl's gyrus and anterior lateral regions) but not surrounding belt regions (planum temporale or anterior medial areas) showed dependencies on both sound level tolerance and tinnitus. The authors note that their results were limited to individuals with mild hyperacusis "…because hyperacusis was never the primary complaint among tinnitus patients recruited for this study and was self-recognized by only a few of the subjects who ultimately showed abnormal sound level tolerance under the controlled conditions of our testing (p. 3368)." Therefore, one must consider the possibility that hyperacusis, which is commonly associated with tinnitus (e.g., Baguley, 2003; Baguley and Andersson, 2007), could be related to the altered FA in individuals with noise-induced tinnitus. While hyperacusis was not studied specifically in the current investigation, further scrutiny in this area is warranted.

### 5.4. Issues regarding the control condition

Issues related to the control condition are relevant to the interpretation of these data. As we indicated in the Introduction and Method sections, in order to examine the effects of tinnitus in those individuals with NIHL, we matched participants as closely as possible for degree of hearing loss and compared groups with and without tinnitus. However, in the limited number of studies evaluating the effects of sensorineural hearing loss on brainstem and cortical white matter tracts, investigators have used a control group with "normal" hearing sensitivity. In this context, Lin et al. (2008) found the FA values were *reduced* in comparison to controls (no hearing loss group) at the level of the lateral lemniscus and inferior colliculus ($N = 37$ sensorineural hearing loss [mean age 32.4years; ±11.9 years]) 10 age-matched controls (mean age 31.1 years; ±11.6 years). In an alternative analysis, Chang et al. (2004) found that FA was *reduced* in one of five regions studied (superior olivary nucleus, inferior colliculus, trapezoid body, lateral lemniscus and auditory radiations) in sensorineural hearing loss (8/10 being binaural). In this sample, the inferior colliculus was most vulnerable to FA anomalies.

In subjects with unilateral cochlear nerve deficiency (aplasia and hypoplasia), Wu et al. (2009b) found significantly *decreased* FA on both ipsilateral and contralateral lateral lemnisci and inferior colliculus which could be attributed to transsynaptic axonal atrophy of these brainstem auditory structures. In long-term unilateral sensorineural hearing loss and using a ROI analysis (lateral lemniscus and inferior colliculus), Wu et al. (2009a) found *decreased* FA on the side contralateral to hearing loss and attributed these effects to "axonal loss and/or demyelination."

Interpretive issues could potentially arise if areas of decreased FA in the group of NIHL without tinnitus were similar in location to those sites where increases in FA were observed. Clearly, this was not the case in our study but it could potentially become an issue in other investigations.

What remains unanswered from the current study is: (1) Which fiber tracts demonstrating neuroplastic changes are directly related to the generation of tinnitus? (2) Which fiber tracts are related to the auditory perception of tinnitus? and (3) Which are related to the presumed emotional/limbic response to this chronic, unpleasant percept?

The answer to question 1 needs to be addressed by future studies. With respect to question 2, in individuals with tinnitus, data from positron emission tomography (PET) studies have shown increased metabolic activity over auditory cortical areas of the left hemisphere in the majority of individuals studied (82%, 9/11) (Arnold et al., 1996) that may be related to our left lateralized results. However, the exact nature of this relationship remains to be determined. With respect to question 3, while these specific white-matter tracts can be considered default pathways when viewed in the context of cortical connectivity of the normal brain, we speculate that some of the pathological changes in FA may be related to the fronto-parietal-cingulate network of brain activations observed in highly distressed people with tinnitus (Golm et al., 2012) and/or as part of a more generalized fear/anxiety/emotion network (Etkin et al., 2011). In fact, the left hemisphere increased metabolic activity

[1] Williams-Beuren syndrome is a multisystem neurodevelopmental genetic disorder with clinical features that include: ear, nose, and throat, cardiovascular, endocrine, dental, gastrointestinal/weight related, genitourinary, and other miscellaneous features (see Pober, 2010 for a medical systems overview). Neuropsychological/cognitive features often show deficits in visual-spatial processing, processing of human faces, lack of fear of strangers, inappropriate friendliness, preserved language abilities, interest and potential aptitude in musical abilities.

demonstrated in PET studies noted above is the basis for the experimental use of low frequency repetitive transcranial magnetic stimulation directed to the left temporoparietal cortex to reduce the tinnitus perception itself (e.g., Piccarillo et al., 2011) and to the left prefrontal cortex to reduce the distress-related components of the tinnitus perception (e.g., Vanneste and De Ridder, 2012). While degree of emotional distress was not studied specifically in the current investigation, further scrutiny in this area is warranted.

## 6. Conclusions

This is the first report demonstrating increased FA in individuals with noise-induced tinnitus using well-matched controls. The foci observed were asymmetric and lateralized predominantly to left ATR and the SLF and inferior longitudinal fasciculus of the left hemisphere. Increased FA could be attributed to a number of factors, including: increased myelination, decreased axonal diameter, increased packing density, and decreased branching. We emphasize that by limiting the inclusion criteria to individuals with tinnitus severity in the moderate-to-severe range and to a specific etiology, heterogeneity of results is minimized and the ability to interpret results is enhanced.

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## References

Aldhafeeri, F.M., Mackenzie, I., Kay, T., Alghamdi, J., Sluming, V., 2012. Neuroanatomical correlates of tinnitus revealed by cortical thickness analysis and diffusion tensor imaging. Neuroradiol. 54, 883–892.
Arlinghaus, L.R., Thornton-Wells, T.A., Dykens, E.M., Anderson, A.W., 2011. Alterations in diffusion properties of white matter in Williams syndrome. Mag. Res. Imag. 29, 1165–1174.
Arnold, W., Bartenstein, P., Oestreicher, E., Römer, W., Schwaiger, M., 1996. Focal metabolic activation in the predominant left auditory cortex in patients suffering from tinnitus: a PET study with [18F] deoxyglucose. ORL J. Otorhinolaryngol. Relat. Spec. 58, 195–199.
Attias, J., Raveh, E., Farber Ben-Naftali, N., Zarchi, O., Gothelf, D., 2008. Hyperactive auditory efferent system and lack of acoustic reflexes in Williams syndrome. J. Basic Clin. Physiol. Pharmacol. 19, 193–207.
Awasthi, R., Gupta, R.K., Trivedi, R., Singh, J.K., Paliwal, V.K., Rathore, R.K.S., 2010. Diffusion tensor MRI imaging in children with pantothenate kinase-associated neurodegeneration with brain iron accumulation and their siblings. Am. J. Neuroradiol. 31, 442–447.
Baguley, D.M., 2003. Hyperacusis. J. R. Soc. Med. 96, 582–585.
Baguley, D.M., Andersson, G., 2007. Hyperacusis. Plural Publishing, Inc., San Diego.
Beaulieu, C., 2002. The basis of anisotropic water diffusion in the nervous system – a technical review. NMR Biomed. 15, 435–455.
Behrens, T.E., Woolrich, M.W., Jenkinson, M., Johansen-Berg, H., Nunes, R.G., Clare, S., Matthews, P.M., Brady, J.M., Smith, S.M., 2003. Characterization and propagation of uncertainty in diffusion-weighted MR imaging. Magn. Reson. Med. 50, 1077–1088.
Boyen, K., Langers, D.R., de Kleine, E., van Dijk, P., 2013. Gray matter in the brain: differences associated with tinnitus and hearing loss. Hear. Res. 295, 67–78.
Brozoski, T., Odintsov, B., Bauer, C., 2012. Gamma-aminobutyric acid and glutamic acid levels in the auditory pathway of rats with chronic tinnitus: a direct determination using high resolution point-resolved proton magnetic resonance spectroscopy (H-MRS). Front. Syst. Neurosci. 6, 1–12.
Browne, C.J., Morley, J.W., Parsons, C.H., 2012. Tracking the expression of excitatory and inhibitory neurotransmission-related proteins and neuroplasticity markers after noise induced hearing loss. PLoS ONE 7, e33272.
Catani, M., Howard, R.J., Pajevic, S., Jones, D.K., 2002. Virtual in vivo interactive dissection of white matter fasciculi in the human brain. NeuroImage 17, 77–94.
Chang, Y., Lee, S.-H., Lee, Y.-J., Hwang, M.-J., Bae, S.-J., Kin, M.-N., Lee, J., Woo, S., Lee, H., Kang, D.-S., 2004. Auditory neural pathway evaluation on sensorineural hearing loss using diffusion tensor imaging. NeuroReport 15, 1699–1703.
Chang, S.-E., Synnestvedt, A., Ostuni, J., Ludlow, C.L., 2010. Similarities in speech and white matter characteristics in idiopathic developmental stuttering and adult-onset stuttering. J. Neurolinguist. 23, 455–469.
Clark, W.W., Bohne, B.A., 1999. Effects of noise on hearing. J. Am. Med. Assoc. 281, 1658–1659.
Coles, R., 2000. Medicolegal issues. In: Tyler, R. (Ed.), Tinnitus Handbook. Singular Publishing Co., San Diego, pp. 399–417.
Crippa, A., Lanting, C.P., van Dijk, P., Roerdink, J.B., 2010. A diffusion tensor imaging study on the auditory system and tinnitus. Open Neuroimag. J. 4, 16–25.
Dong, S., Mulders, W.H., Rodger, J., Woo, S., Robertson, D., 2010. Acoustic trauma evoked hyperactivity and changes in gene expression in guinea-pig auditory brainstem. Eur. J. Neurosci. 31, 1616–1628.
Douaud, G., Behrens, T.E., Poupon, C., Cointepas, Y., Jbabdi, S., Gaura, V., Golestani, N., Krystkowiak, P., et al., 2009. In vivo evidence for the selective subcortical degeneration in Huntington's disease. NeuroImage 46, 958–966.
Eckert, M.A., Galaburda, A.M., Karchemskiy, A., Liang, A., Paul Thompson, P., Dutton, R.A., Lee, A.D., Bellugi, U., Korenberg, J.R., Mills, D., Rose, F.E., Reiss, A.L., 2006. Anomalous Sylvian fissure morphology in Williams syndrome. NeuroImage 33, 39–45.
Eggermont, J.J., Roberts, L.E., 2004. The neuroscience of tinnitus. Trends Neurosci. 27, 676–682.
Elsabbagh, M., Cohen, H., Cohen, M., Rosen, S., Karmiloff-Smith, A., 2011. Severity of hyperacusis predicts individual differences in speech perception in Williams syndrome. J. Intellect. Dis. Res. 55, 563–571.
Etkin, A., Egner, T., Kalisch, R., 2011. Emotional processing in anterior cingulate and medial prefrontal cortex. Trends Cog. Sci. 15, 85–93.
Gattu, R., Benson, R.R., Kou, Z., Zakaria, N., Kallakuri, S., Shen, Y., Cavanaugh, J.M., Haacke, E.M., 2012. Increased FA in acute TBI rat Marmarau model followed by decreased FA during subacute stage: a TBSS study. In: Proc. Int. Soc. Mag. Reson. Med., May 5–11, Melbourne, Australia.
Gerdes, J.S., Keller, S.S., Schwindt, W., Evers, S., Mohammadi, S., Deppe, M., 2012. Progression of microstructural alterations in a case of symptomatic recurrent seizures using diffusion tensor imaging. Seizure 21, 478–481.
Godfrey, D.A., Kaltenbach, J.A., Chen, K., Ilyas, O., Liu, X., Licari, F., Sacks, J., McKnight, D., 2012. Amino acid concentrations in the hamster central auditory system and long-term effects of intense tone exposure. J. Neurosci. Res. 90, 2214–2224.
Golm, D., Schmidt-Samoa, C., Dechent, P., Kröner-Herwig, B., 2012. Neural correlates of tinnitus related distress: an fMRI-study. Hear. Res.. Epub ahead of print.
Gu, J.W., Halpin, C.F., Nam, E.C., Melcher, J.R., 2010. Tinnitus, diminished sound-level tolerance, and elevated auditory activity in humans with clinically normal hearing sensitivity. J. Neurophysiol. 104, 2261–3370.
Harris, M., Gupta, R.K., Hussain, N., Hasan, K.M., Husain, M., Narayana, P.A., 2006. Measurement of DTI metrics in hemorrhagic brain lesions: possible implication in MRI interpretation. J. Mag. Reson. Imag. 24, 1259–1268.
Haas, B.W., Hoeft, F., Barnea-Goraly, N., Golaria, G., Bellugi, U., Reiss, A.L., 2012. Preliminary evidence of abnormal white matter related to the fusiform gyrus in Williams syndrome: a diffusion tensor imaging tractography study. Genes Brain Behav. 11, 62–68.
Haas, B.W., Reiss, A.L., 2012. Social brain development in Williams syndrome: the current status and directions for future research. Front. Psychol. 3, 1–13.
Henderson, D., Bielefeld, E.C., Lobarinas, E., Tanaka, C., 2011. Noise-induced hearing loss: implication for tinnitus. In: Møller, A.R., Langguth, B., De Ridder, D., Kleinjung, T. (Eds.), Textbook of Tinnitus. Springer, New York, pp. 301–309.
Hoeft, F., Barnea-Goraly, N., Haas, B.W., Golarai, G., Ng, D., Mills, D., Korenberg, J., Bellugi, U., Galaburda, A., Reiss, A.L., 2007. More is not always better: increased fractional anisotropy of superior longitudinal fasciculus associated with poor visual spatial abilities in Williams syndrome. J. Neurosci. 27, 11960–11965.
Holinger, D.P., Bellugi, U., Mills, D.L., Korenberg, J.R., Reiss, A.L., Sherman, G.F., Galaburda, A.M., 2005. Relative sparing of primary auditory cortex in Williams Syndrome. Brain Res. 1037, 39–45.
Humes, L.E., Joellenbeck, L.M., Durch, J.S., 2006. Noise and Military Service: Implications for Hearing Loss and Tinnitus. Institute of Medicine of the National Academies, The National Academies Press, Washington, D.C.
Husain, F.T., Medina, R.E., Davis, C.W., Szymko-Bennett, Y., Simonyan, K., Pajor, N.M., Horwitz, B., 2011. Neuroanatomical changes due to hearing loss and chronic tinnitus: a combined VBM and DTI study. Brain Res. 1369, 74–88.
Jiang, H., van Zijl, P.C., Kim, J., Pearlson, G.D., Mori, S., 2006. DtiStudio: resource program for diffusion tensor computation and fiber bundle tracking. Comp. Meth. Progr. Biomed. 81, 106–116.
Kujawa, S.G., Liberman, M.C., 2009. Adding insult to injury: cochlear nerve degeneration after temporary NIHL. J. Neurosci. 29, 14077–14085.
Kaltenbach, J.A., 2011. Tinnitus: models and mechanisms. Hear. Res. 276, 52–60.
Keller, S.S., Ahrens, T., Mohammadi, S., Moddel, G., Kugel, H., Ringelstein, E.B., Deppe, M., 2011. Microstructural and columeric abnormalities of the putamen in juvenile myoclonic epilepsy. Epilepsia 52, 1715–1724.
Klöppel, S., Draganski, B., Golding, C.V., Chu, C., Nagy, Z., Cook, P.A., Hicks, S.L., Kennard, C., Alexander, D.C., Parker, G.J., Tabrizi, S.J., Frackowiak, R.S., 2008. White matter connections reflect changes in voluntary-guided saccades in pre-symtomatic Huntington's disease. Brain 131, 196–204.
Lee, Y.-J., Bae, S.J., Lee, S.H., Lee, J.J., Lee, K.Y., Kim, M.N., Kim, Y.S., Baik, S.K., Woo, S., Chang, Y., 2007. Evaluation of white matter structures in patients with tinnitus using diffusion tensor imaging. J. Clin. Neurosci. 14, 515–519.
Levitan, D.J., Bellugi, U., 1998. Musical abilities in individuals with Williams syndrome. Music. Percept. 15, 357–389.
Levitin, D.J., Menon, V., Schmitt, J.E., Eliez, S., White, C.D., Glover, G.H., Kadis, J., Korenberg, J.R., Bellugi, U., Reiss, A.L., 2003. Neural correlates of auditory perception in Williams Syndrome: an fMRI study. NeuroImage 18, 74–82.

Levitin, D., Cole, K., Lincoln, A., Bellugi, U., 2005. Aversion, awareness, and attraction: investigating claims of hyperacusis in the Williams syndrome phenotype. J. Child Psychol. Psychiatry 46, 514–523.
Li, Q., Sun, J., Guo, L., Zang, Y., Feng, Z., Huang, X., Yang, H., Lv, Y., Huang, M., Gong, Q., 2010. Increased fractional anisotropy in white matter of the right frontal region in children with attention-deficit/hyperactivity disorder: a diffusion tensor imaging study. Neuro-Endocrinol. Lett. 31, 747–753.
Liberman, M.C., Kiang, N.,Y.-S., 1978. Acoustic trauma in cats. Cochlear pathology and auditory-nerve activity. Acta Otolaryngol. Suppl. 358, 1–63.
Lin, Y., Wang, J., Wu, C., Wai, Y., Yu, J., Ng, S., 2008. Diffusion tensor imaging of the auditory pathway in sensorineural hearing loss: changes in radial diffusivity and diffusion anisotropy. J. Mag. Reson. Imag. 28, 598–603.
Ling, J.M., Pena, A., Yeo, R.A., Merideth, F.L., Klimaj, S., Gasparovic, C., Mayer, R., 2012. Biomarkers of increased diffusion anisotropy in semi-acute mild traumatic brain injury: a longitudinal perspective. Brain 135, 1281–1292.
Lockwood, A.H., Salvi, R.J., Burkard, R.F., 2002. Tinnitus. N. Engl. J. Med. 347, 904–910.
Makris, N., Kennedy, D.N., McInerney, S., Sorensen, A.G., Wang, R., Caviness Jr., V.S., Oandya, D.N., 2005. Segmentation of subcomponents within the superior longitudinal facicle in humans: a quantitative, in vivo, DT-MRI study. Cereb. Cortex 15, 854–869.
Mahoney, C.J., Rohrer, J.D., Goll, J.C., Fox, N.C., Rossor, M.N., Warren, J.D., 2011. Structural neuroanatomy of tinnitus and hyperacusis in semantic dementia. J. Neurol. Neurosurg. Psychiatry 82, 1274–1278.
Mamah, D., Conturo, T.E., Harms, M.P., Akbudak, E., Wang, L., McMichael, A.R., Gado, M.H., Barch, D.M., Csernansky, J.G., 2010. Anterior thalamic radiation integrity in schizophrenia: a diffusion tensor imaging study. Psychiatry Res. 183, 144–150.
Matsumoto, N., Kitani, R., Kalinec, F., 2011. Linking LIMK1 deficiency to hyperacusis and progressive hearing loss in individuals with Williams syndrome. Commun. Integr. Biol. 4, 208–210.
Mazurek, B., Olze, H., Haupt, H., Szczepek, A.J., 2010. The more the worse: the grade of noise-induced hearing loss associates with the severity of tinnitus. Int. J. Environ. Res. Pub. Health 7, 3071–3079.
Miani, C., Passon, P., Bracale, A.M., Barotti, A., Panzolli, N., 2001. Treatment of hyperacusis in Williams syndrome with bilateral conductive hearing loss. Eur. Arch. Otorhinolaryngol. 258, 341–344.
Middleton, J.W., Kiritani, T., Pedersen, C., Turner, J.G., Shepherd, G.M., Tzounopoulos, T., 2011. Mice with behavioral evidence of tinnitus exhibit dorsal cochlear nucleus hyperactivity because of decreased GABAergic inhibition. Proc. Natl. Acad. Sci. U S A 108, 7601–7606.
Morest, D.K., Kim, J., Potashner, S.J., Bohne, B.A., 1998. Long-term degeneration in the cochlear nerve and cochlear nucleus of the adult chinchilla following acoustic overstimulation. Microscop. Res. Technol. 41, 205–216.
Mulders, W.H.A.M., Robertson, D., 2011. Progressive centralization of midbrain hyperactivity after acoustic trauma. Neurosci. 192, 753–760.
Mulders, W.H.A.M., Ding, D., Salvi, R., Robertson, D., 2011. Relationship between auditory thresholds, central spontaneous activity, and hair cell loss after acoustic trauma. J. Comp. Neurol. 519, 2637–2647.
Newman, C.W., Sandridge, S.A., 2004. Tinnitus questionnaires. In: Snow Jr., J.B. (Ed.), Tinnitus: Theory and Management. B. C. Decker, Inc., Hamilton, pp. 237–254.
Newman, C.W., Jacobson, G.P., Spitzer, J.B., 1996. Development of the tinnitus handicap inventory. Arch. Otolaryngol. Head Neck Surg. 122, 143–148.
Nigam, A., Samuel, P.R., 1994. Hyperacusis in Williams syndrome. J. Laryng. Otol. 08, 494–496.
Piccarillo, J.F., Garcia, K.S., Nicklaus, J., Pierce, K., Burton, H., Vlassenko, A.G., et al., 2011. Low-frequency repetitive transcranial magnetic stimulation to the temporoparietal junction for tinnitus. Arch. Otolaryngol. Head Neck Surg. 137, 221–228.
Pober, B.R., 2010. Williams-Beuren syndrome. N. Eng. J. Med. 62, 239–252.
Reiss, A., Eckert, M.A., Rose, F.E., Karchemskiy, A., Kesler, S., Chang, M., Reynolds, M.F., Kwon, H., Galaburda, A., 2004. An experiment of nature: brain anatomy parallels cognition and behavior in Williams syndrome. J. Neurosci. 24, 5009–5015.
Roberts, L.E., Eggermont, J.J., Caspary, D.M., Shore, S.E., Melcher, J.R., Kaltenbach, J.A., 2010. Ringing ears: the neuroscience of tinnitus. J. Neurosci. 30, 14972–14979.
Rueckert, D., Sonoda, L.I., Hayes, C., Hill, D.L., Leach, M.O., Hawkes, D.J., 1999. Non-rigid registration using free form deformations: application to breast MR images. IEEE Trans. Med. Imaging 18, 712–721.
Rugg-Gunn, F.J., Eriksson, S.H., Symms, M.R., Barker, G.J., Duncan, J.S., 2001. Diffusion tensor imaging of cryptogenic and acquired partial epilepsies. Brain 124, 627–636.
Salvi, R.J., Wang, J., Ding, D., 2000. Auditory plasticity and hyperactivity following cochlear damage. Hear. Res. 147, 261–274.
Schreiner, C.E., Cheung, S.W., 2004. Cortical plasticity and tinnitus. In: Snow, J.B. (Ed.), Tinnitus: Theory and Management. B. C. Decker, Hamilton, pp. 189–202.
Schecklmann, M., Lehner, A., Poeppl, T.B., Kreuzer, P.M., Rupprecht, R., Rackl, J., Burger, J., Frank, E., Hajak, G., Langguth, B., Landgrebe, M., 2013. Auditory cortex is implicated in tinnitus distress: a voxel-based morphometry study. Brain Struct. Funct. 218, 1061–1070.
Scholz, J., Klein, M.C., Behrens, T.E., Johansen-Berg, H., 2009. Training induces changes in white-matter architecture. Nat. Neurosci. 12, 1370–1371.
Smith, S.M., Jenkinson, M., Woolrich, M.W., Beckmann, C.F., Behrens, T.E.J., Johansen-Berg, H., 2004. Advances in functional and structural MR image analysis and implementation as FSL. NeuroImage 23, 208–219.
Smith, S.M., Jenkinson, M., Johansen-Berg, H., Rueckert, D., Nichols, T.E., Mackay, C.E., Watkins, K.E., Ciccarelli, O., Cader, M.Z., Matthews, P.M., Behrens, T.E., 2006. Track-based spatial statistics: voxelwise analysis of multi-subject diffusion data. NeuroImage 31, 1487–1505.
Snook, L., Plewes, C., Beaulieu, C., 2007. Voxel based versus region of interest analysis in diffusion tensor imaging of neurodevelopment. NeuroImage 34, 243–252.
Steele, C.J., Bailey, J.A., Zattore, R.J., Penhune, V.B., 2013. Early musical training and white-matter plasticity in the corpus callosum: evidence for a sensitive period. J. Neurosci. 33, 1282–1290.
Syka, J., 2002. Plastic changes in the central auditory system after hearing loss, restoration of function, and during learning. Physiol. Rev. 82, 601–636.
Tzouio-Mazoyer, N., Landeau, B., Papathanassiou, D., Crivello, F., Etard, O., Delcroix, N., et al., 2002. Automated anatomical labeling of activations in spm using macroscopic anatomical parcellation of the MNI MRI single subject brain. NeuroImage 15, 273–289.
Vanneste, S., De Ridder, D., 2012. The involvement of the left ventrolateral prefrontal cortex in tinnitus: a TMS study. Exp. Brain Res. 221, 345–350.
Versace, A., Almeida, J.R.C., Hassel, S., Walsh, N.D., Novelli, M., Klein, C.R., Kupfer, D.J., Phillips, M.L., 2008. Elevated left and reduced right orbitomedial prefrontal fractional anisotropy in adults with bipolar disorder revealed by tract-based spatial statistics. Arch. Gen. Psychiatry 65, 1041–1052.
Wang, H., Brozoski, T.J., Caspary, D.M., 2011. Inhibitory neurotransmission in animal models of tinnitus: maladaptive plasticity. Hear. Res. 279, 111–117.
Wu, C.M., Ng, S.H., Liu, T.C., 2009a. Diffusion tensor imaging of the subcortical auditory tract in subjects with long-term unilateral sensorineural hearing loss. Audiol. Neuro-Otol. 14, 248–253.
Wu, C.M., Ng, S.H., Wang, J.J., Liu, T.C., 2009b. Diffusion tensor imaging of the subcortical auditory tact in subjects with congenital cochlear nerve deficiency. Am. J. Neuroradiol. 30, 1773–1776.
Yankaskas, K., 2012. Prelude: noise-induced tinnitus and hearing loss in the military. Hear. Res. 295, 3–8.
Yu, C., Shu, N., Li, J., Qin, W., Jiang, T., Li, K., 2007. Plasticity of the corticospinal tract in early blindness revealed by quantitative analysis of fractional anisotropy based on diffusion tensor tractography. NeuroImage 36, 411–417.
Zhou, S.Y., Suzuki, M., Hagino, H., Takahashi, T., Kawasaki, Y., Nohara, S., Yamashita, I., Seto, H., Kurachi, M., 2001. Decreased volume and increased asymmetry of the anterior limb of the internal capsule in patients with schizophrenia. Biol. Psychiatry 54, 427–436.

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# Blast Induced Tinnitus and Spontaneous Firing Changes in the Rat Dorsal Cochlear Nucleus

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Running head: Blast-induced tinnitus and activity changes

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## Abstract

Exposure to high-pressure blast shock waves is known to cause tinnitus. Although the underlying mechanisms may involve damage to structures in the ear and/or direct brain impact, which triggers a cascade of neuroplastic changes in both auditory and non-auditory centers, it remains unclear how the induced changes manifest in neural activity. In this study, we investigated the influence of blast exposure on spontaneous firing rates in the dorsal cochlear nucleus (DCN) and its time course in rats with blast-induced tinnitus. Each rat was exposed to a single blast at 22 psi. Behavioral evidence of tinnitus was measured using an acoustic startle reflex paradigm. Spontaneous firing rates were measured at one day, one month and three months after blast exposure. The results showed that rats with blast-induced tinnitus developed hyperactivity immediately and at one month after blast exposure. At three months following blast exposure, however, the induced hyperactivity shifted to hypoactivity. In addition, the low frequency region in the DCN of tinnitus positive rats was more affected than other frequency regions at one day and one month after blast exposure, whereas at three months, the DCN was broadly affected except for the low frequency region. These results demonstrated that the neural mechanisms underlying blast-induced tinnitus are much more complex than those underlying noise-induced tinnitus.


*Keywords: Blast exposure, Tinnitus, Traumatic brain injury (TBI), Dorsal cochlear nucleus, Spontaneous activity*

## Introduction

Tinnitus, a noise perception in the absence of an external physical sound, affects an estimated 50 million Americans, of which approximately 18.5 million are afflicted with chronic tinnitus (Heller 2003). Recently, it has been reported that 71% of soldiers in Operation Iraqi Freedom experienced loud noises, and that 15.6% developed tinnitus (Geckle and Lee 2004). Tinnitus is even more prevalent in military personnel with traumatic brain injury (TBI), with up to 38% experiencing comorbid tinnitus (Lew et al. 2007). This suggests that the etiology of tinnitus may be linked to TBI-related plastic changes in the brain (Cave et al. 2007a; Mao et al. 2012). Indeed, blast-related TBI has become a "signature injury" in war and, along with certain civilian situations, frequently leads to tinnitus and hearing loss (Axelsson and Sandh 1985; Cave et al. 2007b; Elder and Cristian 2009). However, due to limited understanding of the underlying neural mechanisms of blast-induced tinnitus, it is difficult to develop effective treatment strategies.

In a previous study, we used diffusion tensor imaging to reveal significant blast-induced microstructural changes including alterations to axonal and myelin integrity in auditory brain regions, with the majority of changes occurring in the inferior colliculus and medial geniculate body (Mao et al. 2012). These results suggest that damage and compensatory plastic changes were incurred in the auditory brain structures, which may be correlated with the presence of tinnitus. We also used manganese-enhanced magnetic resonance imaging to demonstrate increased blast-induced manganese accumulation in the dorsal cochlear nucleus (DCN), ventral cochlear nucleus, medial geniculate body and auditory cortex (in preparation). These findings are generally in line with electrophysiological studies that have shown that noise-induced tinnitus

results from maladaptive plasticity (Eggermont and Roberts 2004; Milbrandt et al. 2000). This plasticity often takes the form of increased spontaneous firing rate (SFR) (hyperactivity) (Manzoor et al. 2013; Mulders et al. 2011; Norena and Eggermont 2003; Vogler et al. 2011; Zhang and Kaltenbach 1998), increased bursting and synchrony, and/or (Ma et al. 2006; Norena and Eggermont 2003), though not all (Langers et al. 2012), altered tonotopic maps along the auditory axis. The affected structures include the DCN (Brozoski et al. 2002; Kaltenbach 2011; Zhang and Kaltenbach 1998; Zhang et al. 2006), ventral cochlear nucleus (Robertson et al. 2012; Vogler et al. 2011), inferior colliculus (Bauer et al. 2008; Ma and Young 2006; Manzoor et al. 2012; Melcher et al. 2009; Mulders and Robertson 2011; Yang et al. 2011) and auditory cortex (Eggermont 2012; Eggermont and Roberts 2004; Llano et al. 2012). However, neurophysiological changes underlying blast-induced tinnitus have not been investigated.

As the first relay station of the central auditory system, the DCN has been regarded as a contributor and/or modulator of noise-induced tinnitus following hyperactivity onset (Brozoski et al. 2002; Kaltenbach 2006; Manzoor et al. 2013; Wallhausser-Franke et al. 2003; Zhang and Kaltenbach 1998). The hyperactivity is believed to be caused by immediate damage to the auditory periphery that triggers plastic changes and reduces intrinsic inhibition (Bauer et al. 2007; Brozoski et al. 2007; Doiron et al. 2011; Kaltenbach 2000; Middleton et al. 2011), such as reduction in inhibitory glycinergic synaptic transmission in the DCN (Richardson et al. 2012; Wang et al. 2009) and upregulation of AMPA receptors in the DCN (Whiting et al. 2009). It has been reported that noise-induced DCN hyperactivity does not occur immediately, but develops several days after exposure (Kaltenbach et al. 2000). The induced hyperactivity gradually increased over a period of 6 months (Kaltenbach et al. 2000), although the frequency distribution across the DCN was broadest at earlier post-exposure time points. Similarly, acoustic trauma-

induced hyperexcitation has been shown to span from the DCN (Manzoor et al. 2013) or ventral cochlear nucleus (Mulders and Robertson 2013) to higher auditory centers. Furthermore, a close relationship has been reported between the 'existence region' of induced hyperactivity and the tonotopic map (Manzoor et al. 2013; Mulders and Robertson 2013). Nevertheless, recent human and animal studies suggest that tinnitus is related to failure of the central auditory pathway to adapt peripheral afferent fiber loss (Gu et al. 2010; Ruttiger et al. 2013; Schaette and Kempter 2012; Singer et al. 2013). One wonders whether blast-induced tinnitus is associated with similar neural activity changes.

In this study, we investigated how blast exposure influences spontaneous activity in the lower auditory brainstem and monitored the time course of blast-induced hyperactivity. Specifically, we sought to establish how hyperactivity emerges after blast exposure and whether the magnitude of the induced hyperactivity shifts over a period of three months. Behavioral evidence of tinnitus was evaluated using a gap-detection acoustic startle reflex paradigm, and hearing detection and thresholds were assessed with prepulse inhibition and auditory brainstem response, respectively. Our results demonstrated tinnitus onset and hyperactivity in the DCN. Such hyperactivity underwent homeostatic changes and transitioned to hypoactivity. This indicates that the DCN has the plastic capacity to embrace a wide scale of changes, and that blast-induced activity changes in the DCN are more complex than those induced by noise exposure.

## Materials and Methods

*Animal subjects*

Forty-two adult male Sprague Dawley rats (70-80 days old at the beginning of experimentation) were purchased from Charles River Laboratories. The rats were assigned into three survival groups: one day, one month and three months after blast exposure. Each group had nine blasted rats and five age-matched control rats. Behavioral testing and ABR recordings were conducted before and after blast exposure, and electrophysiological recordings were performed in the left DCN. For groups that were tested one and three months after blast exposure, behavioral assays were performed on a weekly basis while ABR recordings were conducted monthly until completion of electrophysiological recordings. At the end of experiments, all rats were euthanized and their brains processed histologically to verify electrode location. All experimental procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee at Wayne State University.

*Behavioral testing before and after blast exposure*

Gap-detection (GAP) and pre-pulse inhibition (PPI) testing were used to assess behavioral evidence of tinnitus and hearing status before and after blast exposure. Behavioral testing was conducted with Hamilton-Kinder startle reflex hardware and software (Hamilton-Kinder Behavior Testing System, Poway, CA). As described previously (Turner et al., 2006, Yang et al., 2007; Zhang et al., 2012; Pace and Zhang, 2013), each rat was placed in an individual noise attenuation chamber to perform both GAP and PPI testing. For GAP, each rat was presented with a constant 60 dB SPL background noise, composed of 2000 Hz band-pass signals at 6-8, 10-

12, 14-16, 18-20, or 26-28 kHz, or broadband noise (BBN, 2-30 kHz). The background conditions consisted of 8 startle only trials and 8 trials with a 40 ms silent gap prior to a 50 ms 115 dB broadband noise startle stimulus. The ratio between gap and no gap condition startle amplitude indicated how well the animal could hear the silent gap. PPI procedure and parameters were similar to GAP, except that no background noise or silent gaps were used. For PPI, the startle amplitude of rats in response to two testing conditions was measured: the startle only condition and a pre-pulse followed by the startle stimulus. In the last condition, a 60 dB SPL, 50 ms pre-pulse was introduced 100 ms before the startle stimulus. The rat reduced its acoustic startle reflex in response to the pre-pulse, except when there was hearing loss at a frequency similar to the pre-pulse.

*Auditory brainstem responses (ABRs) before and after blast exposure*

To determine hearing thresholds, click and tone-burst ABRs were conducted before and after blast exposure. Each rat was anesthetized with a mixture of air (1 l/min) and isoflurane (1-2.5% v/v), and was inserted with three sub-dermal platinum electrodes: the active electrode was placed on the top of the head, the reference electrode was inserted below the pinna; and the ground electrode was inserted in the contralateral temporal muscle. Tone burst stimuli at 8, 12, 16, 20, and 28 kHz (0.5 ms rise/fall) and 10 ms in duration were delivered to an electrostatic speaker inserted in the external auditory canal. Each click or tone was presented separately from a 100 dB peak equivalent SPL down to 5 dB in 5 dB decremental steps. The stimuli were generated by an RX6 Multifunction Processor and SigGenRP software (TDT system 3). Tone bursts were calibrated using SigGenRP software. ABR signals were amplified, band filtered (300 Hz-3 kHz) and notch filtered (60 Hz). Click-evoked responses were averaged 300 times and tone

evoked responses 400 times. For analysis, ABR threshold was considered the lowest intensity at which a distinct portion of the biological waveform remained. In addition, the P1-N1 amplitude was calculated to establish input/output functions.

*Blast exposure*

Rats were subjected to a single blast exposure using a custom-designed shock tube (ORA Inc.) located at the Wayne State University Bioengineering Center. Each rat was first anesthetized with a mixture of air (1 l/min) and isoflurane (5% v/v). The rat was then placed on supportive netting with a metal surround and secured on a pole with its head facing the oncoming shockwave. During the blast, the rat's right ear was occluded with a silicone earplug (Mack's®, McKeon Products, Warren, MI) followed by application of mineral oil to seal any remaining openings. The 10 ms blast exposure was estimated to span a wide range of frequencies. The average energy under 10 kHz was measured at 22 psi, which is equivalent to 150 kPa or 197.5 dB SPL. After blast exposure, the rat was transferred to a polycarbonate cage. It was then placed on a water circulating heating pad to prevent hypothermia, and allowed to recover from anesthesia.

*Electrophysiology*

Electrophysiological recordings were performed for all animal groups. Briefly, a rat was first anesthetized with a mixture of air (1 l/min) and isoflurane (5% v/v). The surgical area on its head was then shaven and cleaned. Once no reflex was observed when pinching the hind paw, the rat was placed on a stereotaxic apparatus (Kopf Model 1530) with a pair of custom-made hollow ear bars for sound stimulation. A mixture of air (1 l/min) and isoflurane (1.75-2.5% v/v)

was used to keep the animal unconscious during surgery and recordings. A thermostat-controlled blanket (Harvard Apparatus) was used throughout the procedure to maintain the animal's body temperature at 37 $^{o}$C. Craniotomy was performed to expose the DCN. Briefly, a small piece of occipital bone and underlying dura mater above the left DCN were removed. Following partial aspiration of the cerebellar tissue overhanging the left DCN, its dorsal view was revealed. Using a micromanipulator (Kopf Model 1460-61), a 32-channel electrode probe (NeuroNexus Technologies, Inc.) was inserted into the DCN. The probe had 8 shanks separated by 200 μm (all distances are center to center) and each shank had four recording sites linearly spaced at 50 μm intervals. Each shank was 15 μm thick, 2 mm long, and tapered in width from 33 μm to a few microns at the tip. Prior to insertion, the electrode probe was dipped into a 3% DiI solution (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Invitrogen) prepared with dimethylformamide to label electrode insertion tracks. The probe was inserted to a depth of 150-200 μm below the surface of the DCN, corresponding to the fusiform cell layer (Waller and Godfrey 1994). After probe placement, the brain was covered with agarose to avoid tissue swelling and drying. Spike activity was acquired using a TDT-system 3 (RZ2) data acquisition system. Neural signals were sampled at 25 kHz and filtered at 100-3000 Hz, with threshold set to 1.5 times the standard deviation. Spontaneous single- and multi-unit spikes were recorded twice; one 5 min prior to and one 5 min after performing frequency tuning curve (FTC) construction. Each spontaneous recording period lasted 5 minutes. We used the spontaneous activity data after FTC measures, since they were more stable than those collected before FTC measures. The latter may be due to closer proximity in time to surgical effects.

*Histological verification of electrode position*

At the end of electrophysiological recordings, each rat was euthanized with a lethal dose of isoflurane (5%, v/v) for 15 min and then perfused transcardially with 100 ml saline followed by 300 ml 4% paraformaldehyde. The brain was then removed and post-fixed for 4-6 hours and subsequently cryoprotected by immersion in 30% sucrose in 0.1 M PB (pH 7.4) at 4 °C overnight. Coronal sections were cut on a sliding microtome (HM400, Thermo Scientific.) with the freezing stage (-22 °C) set at a 50 mm thickness. The tracks of electrode positions were verified using a Nikon fluorescent microscope (Eclipse E400).

*Data analysis*

The GAP test was used to monitor tinnitus development. PPI and ABR tests were used to monitor rats' hearing. If a rat had a GAP ratio value above 0.8 at a single frequency band before electrophysiological recordings, it was classified as tinnitus positive. ABR thresholds were used to evaluate hearing loss by comparing the values obtained before blast and one day, one month and three months after blast exposure. In addition to measuring hearing thresholds, the growth functions of P1-N1 amplitudes of each rat's ABR responses were plotted. One-way ANOVA with a post-hoc Bonferroni test was used to determine the group-wise presence of blast-induced tinnitus by comparing tinnitus positive and tinnitus negative rats with age-matched controls. All significant differences were judged using a $p<0.05$ criterion.

Electrophysiologically, in order to plot spontaneous firing rates against tonotopic loci, characteristic frequencies (CFs) were determined from the FTC data by defining a stimulus tone frequency at the lowest intensity level necessary to evoke neural activity. As expected, the FTC data showed that each shank represented a clearly defined CF, and recording shanks reflected the tonotopicity of the DCN with low frequencies represented laterally and high frequencies

represented medially. To avoid misrepresentation of CF-tagged spontaneous firing rates caused by variable penetrations in the DCN across different rats, four frequency bands were created by grouping data at adjacent CFs along the mediolateral axis. Specifically, sites with CFs lower than 10 kHz were grouped to represent a low frequency band, those higher than 10 kHz but lower than 20 kHz were grouped to represent a low-middle frequency band, those higher than 20 kHz but lower than 30 kHz were grouped to represent a middle-high frequency band, and those higher than 30 kHz were grouped to represent a high frequency band.

## Results

After unilateral exposure to a single blast, both behavioral and electrophysiological data were successfully collected from three groups of rats at different recovery times.

*One day after blast exposure*

Behaviorally, as indicated by significant increase in GAP ratio values, the blasted rats showed significantly impaired gap detection compared to non-blasted controls. As can be seen in Figure 1A, GAP ratio values from blasted rats were significantly higher at all frequencies including 6-8 kHz ($F_{(1,127)}$=6.966, $p$<0.01), 10-12 kHz ($F_{(1,125)}$=5.522, $p$<0.01), 14-16 kHz ($F_{(1,127)}$=5.032, $p$<0.01), 18-20 kHz ($F_{(1,127)}$=4.6, $p$<0.05) and 26-28 kHz ($F_{(1,126)}$=2.011, $p$<0.05). Significantly increased PPI ratio values were also found in blast-exposed rats at 6-8 kHz ($F_{(1,123)}$=4.302, $p$<0.05), 10-12 kHz ($F_{(1,127)}$=4.423, $p$<0.05), 14-16 kHz ($F_{(1,126)}$=6.175, $p$<0.01), 18-20 kHz ($F_{(1,123)}$=3.970, $p$<0.05), 26-28 kHz ($F_{(1,123)}$=5.864, $p$<0.05) and broadband noise ($F_{(1,124)}$=4.015, $p$<0.05) (one-way ANOVA test and *post-hoc* Bonferroni tests, Fig. 1A). The

significant increase in GAP ratio values indicated strong evidence of tinnitus at these frequencies and the increase in PPI ratio values indicated compromised auditory detection.

To further examine the impact of blast exposure on hearing, we measured changes in ABR thresholds. As shown in Figure 2, blast exposure induced significant threshold shifts compared to controls in response to both click and tone burst stimuli, with an average threshold shift of approximately 50 dB SPL. The thresholds of unexposed right ears of blasted rats showed no change compared to the non-blasted controls.

Electrophysiologically, we found a significant increase in spontaneous firing rate across the entire mediolateral span of the DCN one day after blast exposure. Specifically, as can be seen in Figure 3A, spontaneous firing rate in blasted rats was increased 1.2 times in the middle-to-high and high frequency regions and increased 1.6 times in the low and low-to-middle frequency regions, compared to the control group. The increase in spontaneous firing rates in blasted rats was statistically significant at four frequency bands spanning a tonotopic range of 2-42 kHz in the DCN: <10 kHz ($F_{(1,110)}$=11.625, $p$<0.01), 10-20 kHz ($F_{(1,111)}$=12.011, $p$<0.01), 20-30 kHz ($F_{(1,108)}$=4.430, $p$<0.05), >30 kHz ($F_{(1,107)}$=4.736, $p$<0.05, one-way ANOVA test and *post-hoc* Bonferroni tests).

*One month after blast exposure*

Behaviorally, one month after blast exposure, our results showed that there were eight tinnitus positive and one tinnitus negative rats. As can be seen in Figure 1B, those tinnitus positive rats had significant increase in GAP ratio values at 14-16 kHz ($F_{(1,127)}$=4.29, $p$=0.037) and 26-28 kHz ($F_{(1,127)}$=10.59, $p$=0.001) (one-way ANOVA and *post-hoc* Bonferroni tests, Fig. 1B). The results indicated that the induced tinnitus tended to be in the middle and high frequency

regions. However, the PPI ratio data from tinnitus positive rats didn't show significant impairments at any frequencies compared to the control group (Fig. 1B). This suggests that the comprised GAP detection responses specifically reflected behavioral evidence of tinnitus.

When examining the impact of blast exposure on hearing threshold, our ABR results showed that the thresholds of the exposed ear, while significantly elevated at 1-day post-blast, returned to control group levels by one month post-blast. The unexposed ears of blasted rats showed no difference compared to the control rats (Fig. 2). Since it has been shown that temporary threshold shift recovery is necessarily indicative of hearing recovery (Kujawa and Liberman 2009), we further examined the impact of blast exposure on these rats by measuring the changes in P1-N1 amplitudes. The results from tinnitus positive rats demonstrated significant degradation in the P1-N1 amplitudes at 14-16 kHz ($F_{(2,165)}=5.745$, $p<0.01$; Fig. 2C) and 26-28 kHz ($F_{(2,166)}=8.731$, $p<0.01$; Fig. 2D), indicating that blast-induced damage to the auditory system persisted after blast exposure.

Electrophysiologically, we found that spontaneous firing rates at one month after blast exposure were also increased, albeit to a smaller degree compared to one day after exposure. Compared to the control group, increased SFRs were found in both tinnitus positive and tinnitus negative groups and were broadly distributed across the entire mediolateral expansion of the DCN. This increase, however, differed from that seen at one day after blast exposure. First, the tinnitus positive rats showed significantly higher SFRs at the <10 kHz and 10-20 kHz regions, but tinnitus negative rats did not show significant difference. The SFRs ratio increased 2.5 times at the <10 kHz region and 1.9 times at the 10-20 kHz region. Second, the tinnitus negative rat showed relatively uniform SFR increases across the four frequency regions, but the increase was slightly higher (1.5 times) in the >30 kHz region than the <10 kHz region (1.3 times) (Fig. 3B).

One-way ANOVA tests showed that the changes were significant at <10 kHz area ($F_{(2,104)}$ =3.237, $p$<0.05), 10-20 kHz ($F_{(2,108)}$ =3.357, $p$<0.05), 20-30 kHz ($F_{(2,106)}$ =0.337, $p$>0.05) and >30 kHz ($F_{(2,107)}$ =0.108, $p$>0.05).

*Three months after blast exposure*

Three months after blast exposure, four rats continued to manifest behavioral evidence of tinnitus while five rats no longer exhibited tinnitus, the latter of which were placed in the tinnitus negative group. Compared to the sham controls, the GAP ratio values of tinnitus positive rats were significantly increased at 6-8 kHz ($F_{(1,127)}$=3.89, $p$<0.05) and 26-28 kHz ($F_{(1,127)}$=6.84, $p$<0.01) (One-way ANOVA test, Fig. 1C). The results appeared to be consistent with the fact that the induced tinnitus occurred at both low and high frequency regions. PPI data didn't show any significant differences at any frequency bands, again suggesting that gap-detection data was not influenced by compromised hearing detection (Fig. 1C).

Consistent with PPI data, ABR data showed no significant differences in hearing thresholds in the blasted left ears and non-blasted right ears compared to controls (Fig. 2). We further examined the impact of blast exposure on hearing by measuring the P1-N1 amplitude at 6-8 kHz and 26-28 kHz of blasted ears of tinnitus positive and negative rats. We found that the P1-N1 amplitude was significantly decreased at 26-28 kHz ($F_{(2,166)}$=8.738, $p$<0.01; Fig. 2E) and no significant difference at 6-8 kHz. This indicated that animals' hearing was still significantly compromised at high-frequencies, even though the threshold recovered.

Electrophysiologically, we found that the patterns of activity changes three months after blast exposure were quite different from those found at one day and one month after blast exposure. Specifically, while tinnitus positive rats showed an increase in spontaneous firing rates

at one day and one month after blast exposure, at three months after blast exposure, they showed decreased SFRs across the entire mediolateral expansion of the DCN. This decrease reached 0.6 times at the >30 kHz, 20-30 kHz and 10-20 kHz regions and 0.9 times in the <10 kHz region. On the contrary, tinnitus negative rats showed increased SFRs across all frequency bands, which became comparable to that seen in the tinnitus negative group at one month after blast exposure. Specially, the increases were 1.6 fold in the 20-30 kHz and >30 kHz regions compared to the sham control group (Fig. 3C). The results were statistically significant at the 10-20 kHz ($F_{(2,102)}$=5.385, $p$<0.01), 20-30 kHz ($F_{(2,104)}$=14.156, $p$<0.01) and >30 kHz ($F_{(2,108)}$=8.672, $p$<0.01).

## Discussion

The foregoing results extended our previous findings that blast exposure induces tinnitus onset and temporary hearing threshold shifts, which were accompanied by neuroplastic changes (Mao et al., 2012). The induced behavioral evidence of tinnitus was accompanied by sustained hyperactivity in the DCN (one day and one month after blast exposure), which transitioned to hypoactivity at 3 months post-blast. These changes indicated continued neural plasticity over time, which is in line with our previous findings (Mao et al. 2012). Blasted rats that did not develop tinnitus, meanwhile, demonstrated delayed hyperactivity at three months after blast exposure. Furthermore, the present results demonstrated that the tonotopic representations of blast-induced hyperactivity in the DCN were different noise-induced hyperactivity (Brozoski et al. 2002; Wallhausser-Franke et al. 2003; Zhang and Kaltenbach 1998), suggesting the involvement of different neural mechanisms.

*Blast induced tinnitus and hearing loss*

The behavioral results confirmed our previous findings that blast-induced early onset tinnitus across all frequency bands, which shifted towards high frequency bands at one month after blast exposure (Mao et al. 2012). These finding are supported by other reports demonstrating that tinnitus tends to occur at high frequency regions (Eggermont and Roberts 2004; Roberts et al. 2010). In addition, our results showed that the current blast exposure parameters induced tinnitus at the 14-16 kHz frequency band one month after exposure and at the 6-8 kHz frequency band three months after exposure. The manifestation of middle frequency tinnitus may result from the fact that the current study used a 22-psi blast whereas our previous study used a 14-psi blast. It is possible that the occurrence of tinnitus at lower frequency regions could be induced by a higher blast intensity that causes more extensive injury to auditory structures, including the DCN. Thus far, no studies have compared the difference in auditory system damage following blast exposure at 14 versus 22 psi. Furthermore, there are no studies to examine the effects of high-level blast exposure (such as 22 psi) on tinnitus and hearing loss. The only available information used to explain and compare our results with come from noise- or intense tone-induced tinnitus models. Although most animal studies showed that noise-induced tinnitus tends to develop at a frequencies higher than the exposure tone (Kraus et al. 2010), it remains unclear what underlies the development of tinnitus at a low or various frequencies as found in the current blast exposure study. Thus far, only one animal study reported tinnitus occurring at 6-24 kHz for up to 10 weeks after intense tone exposure (12 kHz, 120 dB, 2 h) (Kraus et al. 2010). This provides some precedent for blast exposure induced multi-frequency tinnitus.

While chronic tinnitus was induced, only temporary threshold shifts were found following blast exposure, suggesting that a sufficient number of auditory nerve fibers were recruited to maintain normal hearing thresholds (Cho et al. 2013). However, when examining P1-N1 amplitude, we found lasting degradation, which is consistent with previous findings demonstrating that noise-induced shift recovery masks progressive underlying neuropathology (Kujawa and Liberman 2009), especially for high frequency components (Schaette and McAlpine 2011). Thus, our data indicated that the auditory system was indeed impaired at one and three months after blast exposure, even though hearing thresholds recovered. The fact that the P1-N1 amplitude remained to be depressed over a period of three months indicates lasting hearing impairment. Such impairment may subserve the manifestations of tinnitus.

*Blast induced SFR change in the DCN*

The current animal study for the first time delineated the neurophysiological mechanisms underlying blast-induced tinnitus at the auditory brainstem level. As the first relay station along the auditory pathway, the DCN has been regarded as a contributor and/or modulator of tinnitus following intense sound exposure or ototoxic insult (Brozoski et al. 2002; Kaltenbach and McCaslin 1996; Manzoor et al. 2013; Wallhausser-Franke et al. 2003). Previous *in vivo* studies revealed that SFR increased after noise exposure. The induced neural changes vary over time, but are robust and persistent over a long period of time (Kaltenbach et al. 1998; Kaltenbach and McCaslin 1996; Kaltenbach et al. 2000; Kaltenbach et al. 2005). The enhancement of SFR in the DCN may result from loss of peripheral input (Liberman and Kiang 1978; Liberman and Kiang 1984), as blast exposure can directly damage the inner and outer hair cells (Cho et al. 2013; Patterson and Hamernik 1997). Reduced peripheral input may cause loss of inhibition or dis-

inhibition or compensatory enhancement for the lost stimulus-driven activity (Schaette and Kempter 2006). Specifically, cochlear damage may lead to reduced spontaneous firing in the afferent fibers. Through the granule-cartwheel cell circuitry, reduced input from cartwheel cells may diminish inhibitory innervation onto the fusiform cells, causing elevated firing rate from fusiform cells (Kaltenbach et al., 2002). Additionally, an increase in spontaneous and evoked spike rate after acoustic trauma has been link to decreased inhibition (Middleton et al. 2011), reduction in inhibitory glycinergic synaptic transmission in the DCN (Richardson et al. 2012; Wang et al. 2009), and upregulation of AMPA receptors in the DCN (Whiting et al. 2009). Therefore, the observed increased SFRs in rats with tinnitus at one day and one month after blast appeared to be consistent with the results from those previous studies using noise exposures (Kaltenbach and Afman 2000; Kaltenbach et al. 1998; Salvi et al. 2000; Zhang and Kaltenbach 1998). Interestingly, we found in tinnitus negative rats that SFRs increased at one and three months after blast exposure. That fact that the P1-N1 amplitude significantly decreased suggests that hearing loss indeed played a role in blast-induced neural changes. Depending on the homeostatic condition following acoustic trauma, hyperactivity may not necessarily be the only contributing factor to tinnitus generation.

Indeed, as demonstrated by the current results using blast exposure, we found that SFRs in the DCN of tinnitus positive rats significantly decreased, or developed significant hypoactivity, three months after blast exposure. These results appear to be consistent with previous findings from noise exposure experiments (Finlayson and Kaltenbach 2009; Salvi et al. 2000). Mechanistically, the hypoactivity may result from a different neural plasticity process. For instance, the extreme high-pressure blast waves could have caused significant damage to both inner and outer hair cells. The damage could disrupt the basilar membrane, which may cause

secondary damage to the hair cells and supporting cells as evidenced by cellular degeneration in the cochlea (Hoffer et al. 2010; Patterson and Hamernik 1997). Our recent immunostaining results showed sustained axonal injury from one to three months after blast exposure (in preparation), which may have caused a decline in SFRs, especially in SFRs of those hyperactivity-inclined neurons. In addition, the observed hypoactivity suggests that hyperactivity may not necessarily come from all neurons. In other studies, only certain neuron clusters in the DCN became hyperactive following blast exposure (Kaltenbach 2011). Similarly, it has been reported that noise lesion could lead to a number of plastic changes in the cochlear nucleus, including widespread growth of new fibers and degeneration of pre-exiting fibers (Bilak et al. 1997; Kim et al. 1997); the presence of new fibers has been observed until 2 months after exposure (Bilak et al. 1997). This delay may help explain why SFRs in the DCN of tinnitus positive rats was only lower than controls at three months after blast exposure. Furthermore, the DCN is known to receive neural input from both lemniscal and non-lemniscal systems (Malmierca et al. 2002; Mcintosh and Gonzalezlima 1993). This being said, the extreme physical force of the blast exposure may not only damage hearing, but may also directly cause TBI and induced neuroplasticity (Elder and Cristian 2009; Povlishock et al. 1992). It is conceivable that TBI-induced neuroplasticity may also be responsible for the etiology of the tinnitus. Neurophysiologically, previous studies suggest that tinnitus generation may be integrated with a global neural network through cortical connectivity with other structures (Eggermont and Roberts 2004; Roberts et al. 2010). Finally, previous studies on tinnitus suggest that neural activity within the middle brain, thalamus, and cortex, may be readjusted for new gain control (Knipper et al. 2013; Mulders et al. 2011; Mulders and Robertson 2011). After blast exposure, the effects of TBI and neuroplasticity at different brain centers may vary in order to achieve the

needed gating adjustment for gain control (Zhang 2013). From our recent parallel studies, the induced hyperactivity in the auditory cortex of tinnitus positive rats occurred at one month after blast exposure (in preparation). Additional information is needed, however, to further delineate the neural activity changes observed after blast exposure.

Our results showed significant SFR changes in tinnitus positive rats at all frequency loci one day after blast exposure. SFR changes were also observed at low and low-to-middle frequency loci at one month after blast exposure and at low-to-middle, middle, middle-to-high frequency loci at three months after blast exposure. This is not the same as findings from studies using noise exposure. For example, noise-induced hyperactivity was broadly distributed across the DCN at early time points (5 and 14 days), and then later at medial positions (30 and 180 days) (Kaltenbach et al. 2000). The disparity between these results and those of our study may come from the extreme intensity of blast exposure, which could broadly damage the inner and outer hair cells at the same time, causing injury to the entire frequency expansion whereas noise exposure tends to cause injury to the high frequency region (Liberman and Beil 1979; Patterson and Hamernik 1997). After three months, neuroplasticity may cause the frequency loci of induced SFR to change from low to high frequency. This frequency loci shift also suggests that blast-induced tinnitus may come from certain neuronal activity changes at different time courses after blast exposure.

Over the past decade, there has been a rapid increase in scientific interest in the neurobiological origins of tinnitus. The converging view is that tinnitus is a systemic problem generated by an imbalance in the excitatory and inhibitory inputs to the auditory system (Eggermont and Roberts 2004; Roberts et al. 2010). Such changes occur at multiple levels of the auditory system and involve a combination of interactive factors that are initiated by loss of

peripheral input from the inner ear. From our results, the SFR changes were different compared to animal studies utilizing noise exposure, suggesting that blast induced-tinnitus does not necessarily involve a single model change in the auditory system. As shown in the results from the three time points, the tendency of SFR changes in the DCN suggests that complex pathophysiological processes occur along with tinnitus manifestations following blast-exposure. The DCN can sustain hyperactivity immediately after blast exposure, and such hyperactivity may last before decreasing to a lower level comparable to that from control animals at three months after blast exposure. Taken together, the neural mechanisms underlying blast-induced tinnitus are much more complex than those underlying noise-induced tinnitus.



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## Reference

Axelsson A, Sandh A. 1985. Tinnitus in noise-induced hearing loss. BrJAudiol 19(4):271-276.

Bauer CA, Brozoski TJ, Myers K. 2007. Primary afferent dendrite degeneration as a cause of tinnitus. Journal of neuroscience research 85(7):1489-1498.

Bauer CA, Turner JG, Caspary DM, Myers KS, Brozoski TJ. 2008. Tinnitus and inferior colliculus activity in chinchillas related to three distinct patterns of cochlear trauma. Journal of neuroscience research 86(11):2564-2578.

Bilak M, Kim J, Potashner SJ, Bohne BA, Morest DK. 1997. New growth of axons in the cochlear nucleus of adult chinchillas after acoustic trauma. Exp Neurol 147(2):256-268.

Brozoski TJ, Bauer CA, Caspary DM. 2002. Elevated fusiform cell activity in the dorsal cochlear nucleus of chinchillas with psychophysical evidence of tinnitus. J Neurosci 22(6):2383-2390.

Brozoski TJ, Spires TJ, Bauer CA. 2007. Vigabatrin, a GABA transaminase inhibitor, reversibly eliminates tinnitus in an animal model. J Assoc Res Otolaryngol 8(1):105-118.

Cave KM, Cornish EM, Chandler DW. 2007a. Blast injury of the ear: clinical update from the global war on terror. Mil Med 172(7):726-730.

Cave KM, Cornish EM, Chandler DW. 2007b. Blast injury of the ear: clinical update from the global war on terror. MilMed 172(7):726-730.

Cho SI, Gao SS, Xia A, Wang R, Salles FT, Raphael PD, Abaya H, Wachtel J, Baek J, Jacobs D, Rasband MN, Oghalai JS. 2013. Mechanisms of hearing loss after blast injury to the ear. PLoS One 8(7):e67618.

Doiron B, Zhao Y, Tzounopoulos T. 2011. Combined LTP and LTD of modulatory inputs controls neuronal processing of primary sensory inputs. The Journal of neuroscience : the official journal of the Society for Neuroscience 31(29):10579-10592.

Eggermont JJ. 2012. Hearing loss, hyperacusis, or tinnitus: What is modeled in animal research? Hear Res.

Eggermont JJ, Roberts LE. 2004. The neuroscience of tinnitus. Trends in neurosciences 27(11):676-682.

Elder GA, Cristian A. 2009. Blast-related mild traumatic brain injury: mechanisms of injury and impact on clinical care. The Mount Sinai journal of medicine, New York 76(2):111-118.

Finlayson PG, Kaltenbach JA. 2009. Alterations in the spontaneous discharge patterns of single units in the dorsal cochlear nucleus following intense sound exposure. Hear Res 256(1-2):104-117.

Geckle L, Lee R. 2004. Soldier perceptions of deployment environmental exposures. Force Health Protection Conference. Albuquerque, NM.

Gu JW, Halpin CF, Nam EC, Levine RA, Melcher JR. 2010. Tinnitus, Diminished Sound-Level Tolerance, and Elevated Auditory Activity in Humans With Clinically Normal Hearing Sensitivity. Journal of Neurophysiology 104(6):3361-3370.

Heller AJ. 2003. Classification and epidemiology of tinnitus. Otolaryngol Clin North Am 36(2):239-248.

Hoffer ME, Balaban C, Gottshall K, Balough BJ, Maddox MR, Penta JR. 2010. Blast exposure: vestibular consequences and associated characteristics. Otol Neurotol 31(2):232-236.

Kaltenbach JA. 2000. Neurophysiologic mechanisms of tinnitus. J Am Acad Audiol 11(3):125-137.

Kaltenbach JA. 2006. The dorsal cochlear nucleus as a participant in the auditory, attentional and emotional components of tinnitus. Hear Res 216-217:224-234.

Kaltenbach JA. 2011. Tinnitus: Models and mechanisms. Hear Res 276(1-2):52-60.

Kaltenbach JA, Afman CE. 2000. Hyperactivity in the dorsal cochlear nucleus after intense sound exposure and its resemblance to tone-evoked activity: a physiological model for tinnitus. Hearing Res 140(1-2):165-172.

Kaltenbach JA, Godfrey DA, Neumann JB, McCaslin DL, Afman CE, Zhang J. 1998. Changes in spontaneous neural activity in the dorsal cochlear nucleus following exposure to intense sound: relation to threshold shift. Hear Res 124(1-2):78-84.

Kaltenbach JA, McCaslin DL. 1996. Increases in spontaneous activity in the dorsal cochlear nucleus following exposure to high intensity sound: A possible neural correlate of tinnitus. Audit Neurosci 3(1):57-78.

Kaltenbach JA, Zhang J, Afman CE. 2000. Plasticity of spontaneous neural activity in the dorsal cochlear nucleus after intense sound exposure. Hear Res 147(1-2):282-292.

Kaltenbach JA, Zhang J, Finlayson P. 2005. Tinnitus as a plastic phenomenon and its possible neural underpinnings in the dorsal cochlear nucleus. Hear Res 206(1-2):200-226.

Kim J, Morest DK, Bohne BA. 1997. Degeneration of axons in the brainstem of the chinchilla after auditory overstimulation. Hearing Res 103(1-2):169-191.

Knipper M, Van Dijk P, Nunes I, Ruttiger L, Zimmermann U. 2013. Advances in the neurobiology of hearing disorders: Recent developments regarding the basis of tinnitus and hyperacusis. Prog Neurobiol.

Kraus KS, Mitra S, Jimenez Z, Hinduja S, Ding D, Jiang H, Gray L, Lobarinas E, Sun W, Salvi RJ. 2010. Noise trauma impairs neurogenesis in the rat hippocampus. Neuroscience 167(4):1216-1226.

Kujawa SG, Liberman MC. 2009. Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. J Neurosci 29(45):14077-14085.

Langers DR, de Kleine E, van Dijk P. 2012. Tinnitus does not require macroscopic tonotopic map reorganization. Front Syst Neurosci 6:2.

Lew HL, Jerger JF, Guillory SB, Henry JA. 2007. Auditory dysfunction in traumatic brain injury. J Rehabil Res Dev 44(7):921-928.

Liberman MC, Beil DG. 1979. Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. Acta Oto-Laryngol 88(3-4):161-176.

Liberman MC, Kiang NY. 1978. Acoustic trauma in cats. Cochlear pathology and auditory-nerve activity. Acta oto-laryngologica Supplementum 358:1-63.

Liberman MC, Kiang NY. 1984. Single-neuron labeling and chronic cochlear pathology. IV. Stereocilia damage and alterations in rate- and phase-level functions. Hear Res 16(1):75-90.

Llano DA, Turner J, Caspary DM. 2012. Diminished cortical inhibition in an aging mouse model of chronic tinnitus. J Neurosci 32(46):16141-16148.

Ma WL, Hidaka H, May BJ. 2006. Spontaneous activity in the inferior colliculus of CBA/J mice after manipulations that induce tinnitus. Hearing Res 212(1-2):9-21.

Ma WL, Young ED. 2006. Dorsal cochlear nucleus response properties following acoustic trauma: response maps and spontaneous activity. Hearing Res 216-217:176-188.

Malmierca MS, Merchan MA, Henkel CK, Oliver DL. 2002. Direct projections from cochlear nuclear complex to auditory thalamus in the rat. J Neurosci 22(24):10891-10897.

Manzoor N, Licari FG, Klapchar M, Elkin R, Gao Y, Kaltenbach JA. 2012. Noise-induced hyperactivity in the inferior colliculus: its relationship with hyperactivity in the dorsal cochlear nucleus. J Neurophysiol.

Manzoor NF, Gao Y, Licari F, Kaltenbach JA. 2013. Comparison and contrast of noise-induced hyperactivity in the dorsal cochlear nucleus and inferior colliculus. Hear Res 295:114-123.

Mao JC, Pace E, Pierozynski P, Kou Z, Shen Y, Vandevord P, Haacke EM, Zhang X, Zhang J. 2012. Blast-induced tinnitus and hearing loss in rats: behavioral and imaging assays. Journal of neurotrauma 29(2):430-444.

Mcintosh AR, Gonzalezlima F. 1993. Network Analysis of Functional Auditory Pathways Mapped with Fluorodeoxyglucose - Associative Effects of a Tone Conditioned as a Pavlovian Excitor or Inhibitor. Brain Research 627(1):129-140.

Melcher JR, Levine RA, Bergevin C, Norris B. 2009. The auditory midbrain of people with tinnitus: abnormal sound-evoked activity revisited. Hearing Res 257(1-2):63-74.

Middleton JW, Kiritani T, Pedersen C, Turner JG, Shepherd GM, Tzounopoulos T. 2011. Mice with behavioral evidence of tinnitus exhibit dorsal cochlear nucleus hyperactivity because of decreased GABAergic inhibition. Proc Natl Acad Sci U S A 108(18):7601-7606.

Milbrandt JC, Holder TM, Wilson MC, Salvi RJ, Caspary DM. 2000. GAD levels and muscimol binding in rat inferior colliculus following acoustic trauma. Hear Res 147(1-2):251-260.

Mulders WH, Ding D, Salvi R, Robertson D. 2011. Relationship between auditory thresholds, central spontaneous activity, and hair cell loss after acoustic trauma. J Comp Neurol 519(13):2637-2647.

Mulders WH, Robertson D. 2011. Progressive centralization of midbrain hyperactivity after acoustic trauma. Neuroscience 192:753-760.

Mulders WH, Robertson D. 2013. Development of hyperactivity after acoustic trauma in the guinea pig inferior colliculus. Hear Res 298:104-108.

Norena AJ, Eggermont JJ. 2003. Changes in spontaneous neural activity immediately after an acoustic trauma: implications for neural correlates of tinnitus. Hearing Res 183(1-2):137-153.

Patterson JH, Jr., Hamernik RP. 1997. Blast overpressure induced structural and functional changes in the auditory system. Toxicology 121(1):29-40.

Povlishock JT, Erb DE, Astruc J. 1992. Axonal Response to Traumatic Brain Injury - Reactive Axonal Change, Deafferentation, and Neuroplasticity. Journal of neurotrauma 9:S189-S200.

Richardson BD, Brozoski TJ, Ling LL, Caspary DM. 2012. Targeting inhibitory neurotransmission in tinnitus. Brain Res 1485:77-87.

Roberts LE, Eggermont JJ, Caspary DM, Shore SE, Melcher JR, Kaltenbach JA. 2010. Ringing ears: the neuroscience of tinnitus. The Journal of neuroscience : the official journal of the Society for Neuroscience 30(45):14972-14979.

Robertson D, Bester C, Vogler D, Mulders WH. 2012. Spontaneous hyperactivity in the auditory midbrain: Relationship to afferent input. Hear Res.

Ruttiger L, Singer W, Panford-Walsh R, Matsumoto M, Lee SC, Zuccotti A, Zimmermann U, Jaumann M, Rohbock K, Xiong H, Knipper M. 2013. The reduced cochlear output and the failure to adapt the central auditory response causes tinnitus in noise exposed rats. PLoS One 8(3):e57247.

Salvi RJ, Wang J, Ding D. 2000. Auditory plasticity and hyperactivity following cochlear damage. Hearing Res 147(1-2):261-274.

Schaette R, Kempter R. 2006. Development of tinnitus-related neuronal hyperactivity through homeostatic plasticity after hearing loss: a computational model. Eur J Neurosci 23(11):3124-3138.

Schaette R, Kempter R. 2012. Computational models of neurophysiological correlates of tinnitus. Front Syst Neurosci 6:34.

Schaette R, McAlpine D. 2011. Tinnitus with a normal audiogram: physiological evidence for hidden hearing loss and computational model. J Neurosci 31(38):13452-13457.

Singer W, Zuccotti A, Jaumann M, Lee SC, Panford-Walsh R, Xiong H, Zimmermann U, Franz C, Geisler HS, Kopschall I, Rohbock K, Varakina K, Verpoorten S, Reinbothe T, Schimmang T, Ruttiger L, Knipper M. 2013. Noise-induced inner hair cell ribbon loss disturbs central arc mobilization: a novel molecular paradigm for understanding tinnitus. Molecular neurobiology 47(1):261-279.

Vogler DP, Robertson D, Mulders WH. 2011. Hyperactivity in the ventral cochlear nucleus after cochlear trauma. The Journal of neuroscience : the official journal of the Society for Neuroscience 31(18):6639-6645.

Waller HJ, Godfrey DA. 1994. Functional characteristics of spontaneously active neurons in rat dorsal cochlear nucleus in vitro. Journal of neurophysiology 71(2):467-478.

Wallhausser-Franke E, Mahlke C, Oliva R, Braun S, Wenz G, Langner G. 2003. Expression of c-fos in auditory and non-auditory brain regions of the gerbil after manipulations that induce tinnitus. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 153(4):649-654.

Wang H, Brozoski TJ, Turner JG, Ling L, Parrish JL, Hughes LF, Caspary DM. 2009. Plasticity at glycinergic synapses in dorsal cochlear nucleus of rats with behavioral evidence of tinnitus. Neuroscience 164(2):747-759.

Whiting B, Moiseff A, Rubio ME. 2009. Cochlear nucleus neurons redistribute synaptic AMPA and glycine receptors in response to monaural conductive hearing loss. Neuroscience 163(4):1264-1276.

Yang S, Weiner BD, Zhang LS, Cho SJ, Bao S. 2011. Homeostatic plasticity drives tinnitus perception in an animal model. Proc Natl Acad Sci U S A 108(36):14974-14979.

Zhang J. 2013. Auditory cortex stimulation to suppress tinnitus: mechanisms and strategies. Hear Res 295:38-57.

Zhang JS, Kaltenbach JA. 1998. Increases in spontaneous activity in the dorsal cochlear nucleus of the rat following exposure to high-intensity sound. Neurosci Lett 250(3):197-200.

Zhang JS, Kaltenbach JA, Godfrey DA, Wang J. 2006. Origin of hyperactivity in the hamster dorsal cochlear nucleus following intense sound exposure. J Neurosci Res 84(4):819-831.



**Figure legends:**

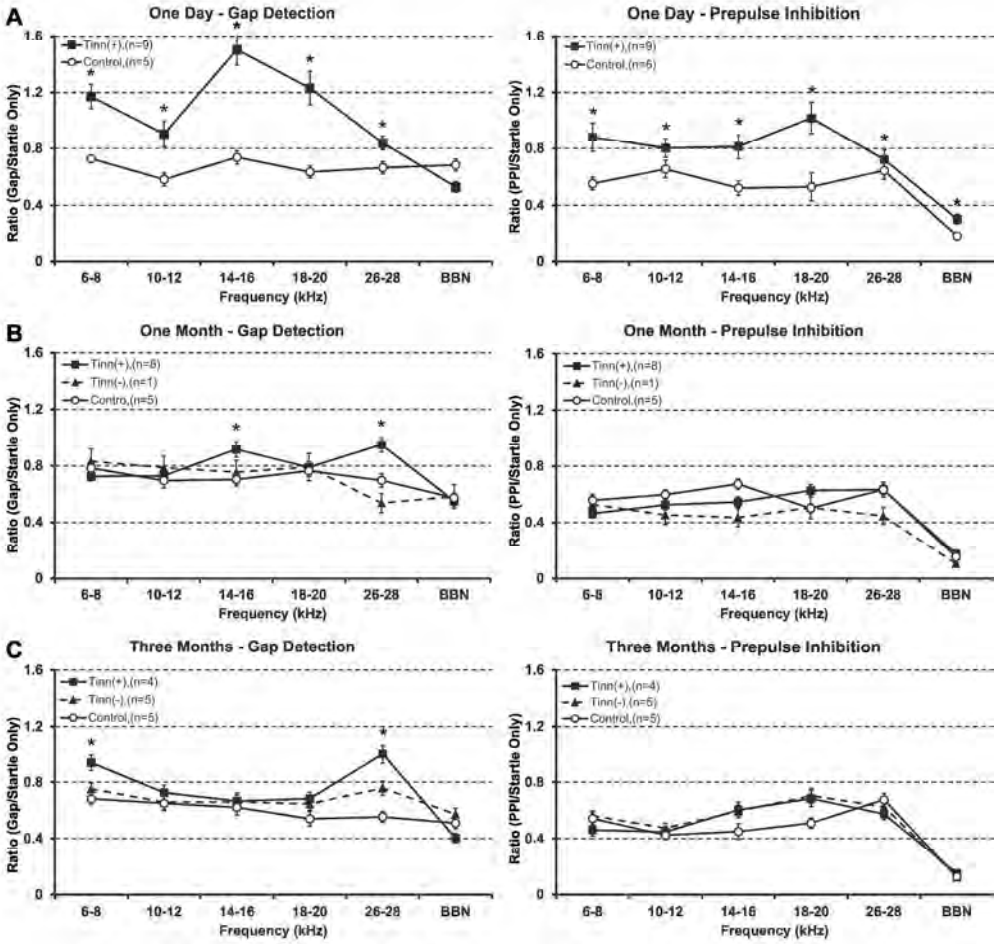
Figure 1. Gap detection ratio values (gap detection/Startle-only response) and PPI ratio values (PPI/startle-only response) measured from tinnitus positive animals, tinnitus negative animals and age-matched control animals, at one day after blast exposure (A), one month after blast exposure (B) and three months after blast exposure (C). Note that rats showed significant deficits in gap-detection and PPI at one day after blast exposure, followed by marked tinnitus at one month after blast exposure in the gap detection test. Further tinnitus spread was found at three months after blast exposure in the gap detection test. Error bars represent standard error of the mean. * $p<0.05$.

Figure 2. ABR thresholds were measured in the left ear (A) and right ear (B) at one day, one month, and three months after blast exposure. ABR thresholds in tinnitus positive animals were significantly elevated at one day after blast exposure, and then returned to control levels at one month and three months after blast exposure (Left panel). P1-N1 amplitudes were measured at 14-16 kHz and 26-28 kHz at one month after blast exposure, and at 26-28

kHz of three months after blast exposure (Right panel). Error bars represent standard error of the mean.

Figure 3. Based on the characteristic frequency recorded at four frequency bands (<10 kHz, 10-20 kHz, 20-30 kHz and >30 kHz), SFR was measured from tinnitus positive animals, tinnitus negative animals and age-matched control animals, at one day, one month, and three months after blast exposure. Error bars represent standard error of the mean. * $p<0.05$.

Figure 1

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356x360mm (300 x 300 DPI)

Figure 2

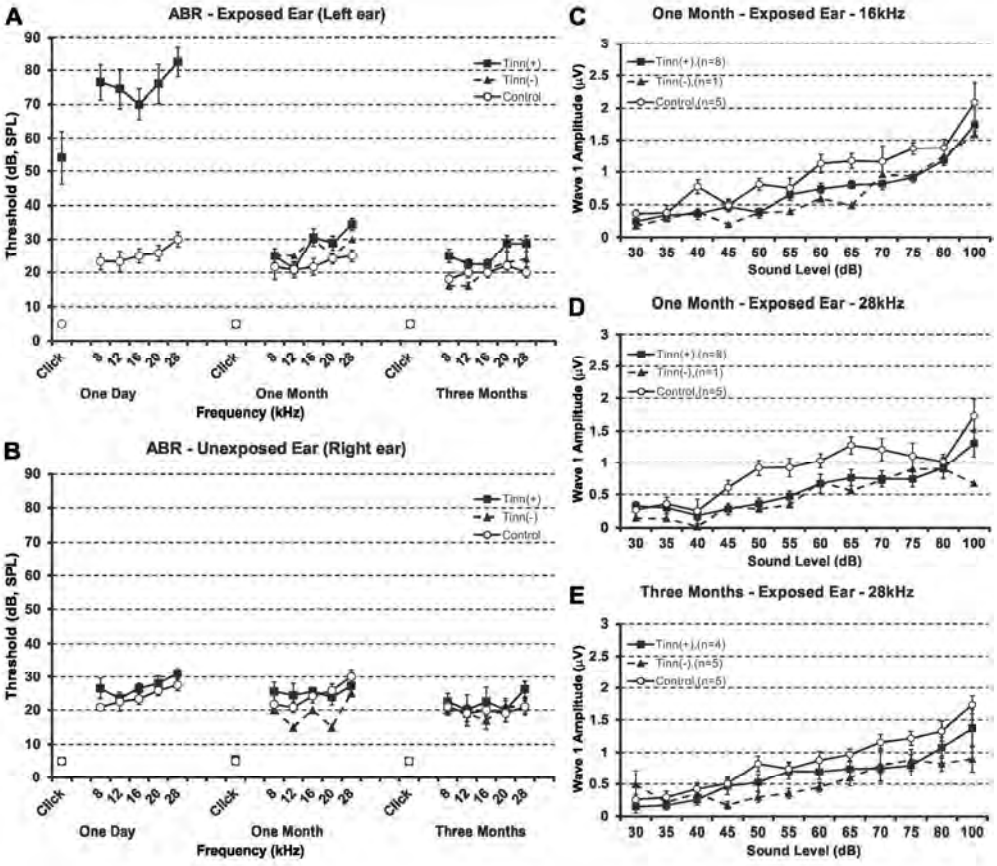


Figure 2. ABR thresholds were measured in the left ear (A) and right ear (B) at one day, one month, and three months after blast exposure. ABR thresholds in tinnitus positive animals were significantly elevated at one day after blast exposure, and then returned to control levels at one month and three months after blast exposure (Left panel). P1-N1 amplitudes were measured at 14-16 kHz and 26-28 kHz at one month after blast exposure, and at 26-28 kHz of three months after blast exposure (Right panel). Error bars represent standard error of the mean.
178x168mm (300 x 300 DPI)

## Figure 3

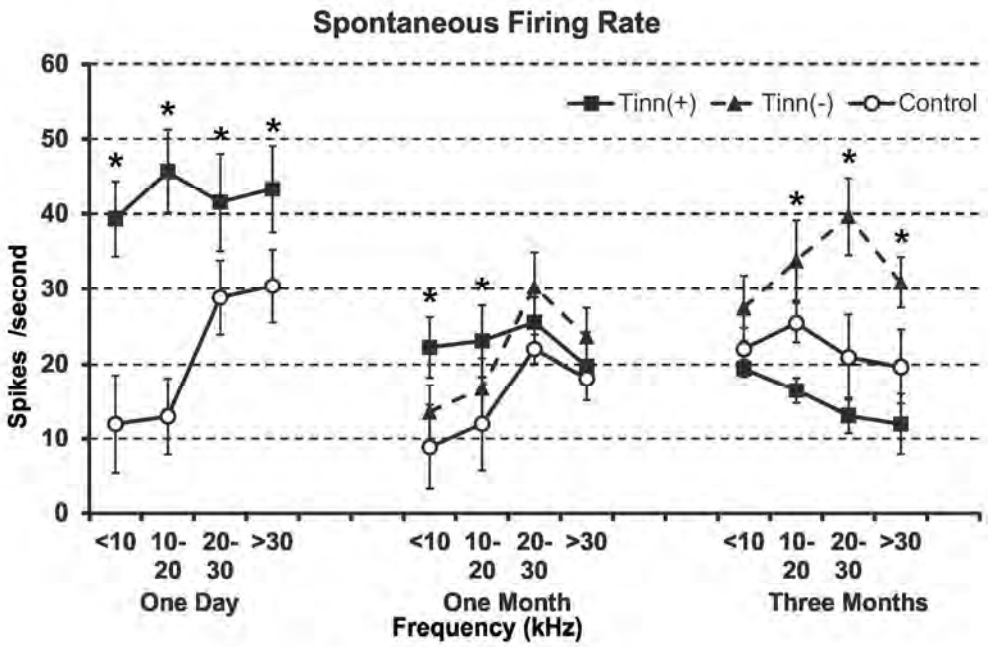


Figure 3. Based on the characteristic frequency recorded at four frequency bands (<10 kHz, 10-20 kHz, 20-30 kHz and >30 kHz), SFR was measured from tinnitus positive animals, tinnitus negative animals and age-matched control animals, at one day, one month, and three months after blast exposure. Error bars represent standard error of the mean. * $p<0.05$.
202x159mm (300 x 300 DPI)

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Manuscript Draft

Manuscript Number: NSC-13-1427R1

Title: Therapeutic Effect of Sildenafil on Blast-Induced Tinnitus and Auditory Impairment

Article Type: Research Paper

Section/Category: Pain Mechanisms and Sensory Neuroscience

Keywords: Tinnitus, Blast, Traumatic brain injury, Sildenafil (Viagra, Revatio), Hyperacusis, Rat

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Order of Authors: Gulrez Mahmood; Zhigang Mei; Houmehr Hojjat; Edward Pace; Jinsheng Zhang

Abstract: Blast-induced tinnitus, along with associated auditory impairment and traumatic brain injury, is a primary concern facing military service members. To search for treatment, we investigated the therapeutic effects of sildenafil, a phosphodiesterase-5 inhibitor, given its vasodilation effects and evidence suggesting its beneficial effects on noise-induced hearing loss. Rats were subjected to three consecutive blast exposures at 22 psi and were monitored for tinnitus using a gap-detection acoustic startle reflex paradigm. Hearing thresholds and detection were tested using auditory brainstem responses and prepulse inhibition, respectively. Blasted rats were either treated with sildenafil or tap water following blast exposure, while age-matched sham control rats were treated with sildenafil without blast exposure. Our results showed that sildenafil did not effectively prevent acute tinnitus onset and hearing impairment. Instead, sildenafil significantly suppressed high-frequency tinnitus from 3 to 6 weeks after blast exposure and reduced hearing impairment during the first week after blast exposure. Complex results were observed in the startle force data, where sildenafil-treated rats displayed significantly reduced startle force compared to the untreated control group, suggesting of possible mitigation of traumatic brain injury and suppression of hyperacusis-like percepts. Taken together, sildenafil showed a therapeutic effect on blast-induced tinnitus and audiological impairment in a time-dependent manner. Other regimen such as higher dosage prior to blast exposure and in combination with other treatments deserves investigations to optimize therapeutic effects.

Response to Reviewers: We thank our reviewers very much for their time and efforts to improve our manuscript. Below are our responses to reviewers' comments.

Responses to Reviewer 1:
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Question 1: …I would take the details in the results section and placed them in table form and then simply refer to the table in the prose. That way the details would be easier to see for he reader.

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Question 5: Furthermore, some weaknesses limit the acceptation of the data. The Sildenafil application was for a first week then there was one week interruption, then another week of treatment. It is not clear why this treatment regime was chosen. This complicates the interpretability of the data.

Response: One full week of treatment was originally our intention, but after seeing a mild therapeutic effect during the second week (washout), we decided to give another week-long dose of the drug to achieve a more robust therapeutic effect. We also wanted to avoid continuously administering sildenafil for too long a period of time, as some report has indicated that PDE5-inhibitors can contribute to hearing loss. We have now added these statements in the Methods sections to clarify. In spite of these aforementioned factors, they do not, in any way, negate the observation that sildenafil provides protection from blast induced auditory damage. Now that we have established the therapeutic potential of sildenafil, we can attempt to identify an optimal treatment regimen, although this should be done in a separate study and discussion that compares different treatment strategies.

Question 6: The statistical presentation is very awkward. Even if the authors used this kind of presentation in previous publications, I doubt it is correct. If 1-way-ANOVA was chosen (this information is not given in the methods) only one single F-value can result from a single group comparison, followed by indication of significance from the pair wise comparison by the Bonferroni post-hoc test. In contrast to this, the authors give an F-value for every single frequency and time point. This may be not correct. If 2-way-ANOVA or 3-way ANOVA had been used (unclear whether it was) the authors must tell which factors / source of variation they refer to when they give the F-value from the ANOVA. Additionally, the time series data are not independent (same animals) and need adjustment for multiple testing by correction of family wise error and/or modeling of multivariant ANOVA (MANOVA). The statistics must be explained.

Response: To clarify our statistical analysis, we indicated that we used one-way ANOVA and post-hoc Bonferroni in the Data Analysis section. In this study, we compared the degree of change (gap/PPI percent change, startle amplitude change, and threshold shifts) between groups at each time point. We did not compare data from different time points within the same group – this type of analysis would not be useful since tinnitus, hearing loss, hyperacusis, and TBI-related effects could themselves change over the course of time and confound sildenafil-treatment effects. If we had compared data across time from within the same group, then yes, we would only have one F value for between group comparisons. However, since we compared between the three groups at each time point, we had an F value for the overall three-group comparison, and post-hoc values for specific between group comparisons.

Question 7: The authors plus one ear to maintain hearing on one ear after blast. I guess this was done since the startle model is not giving reliable and reproducible results if the animals suffer from a general loss of auditory thresholds (this is the precondition the authors explain in their methods part). However, a hearing loss remains on both ears, also on the plugged, desired protected ear. It is unclear how this changes the startle responses (those are then interpreted as signs for tinnitus and hyperacusis).

Response: Although both ears sustained hearing loss through one week post-blast, only the exposed left ear retained significant thresholds shifts beyond this time point. It has been shown that unilateral hearing threshold shifts, even when permanent, do not necessarily alter detectability of the gap or startle responsivity (Turner et al., 2006; Pace and Zhang 2013; Sun et al., 2012; Chen et al, 2013).

Question 8: Startle responses are recently carefully revisited as models for 'hyperacusis', since it is not clear what the various tests are reflecting (tinnitus or hyperacusis, see e.g. JJ Eggermont Hearing Research 2013). The authors themselves agree that their results from the startle experiments are complex and complicated. For a general readership, these results are undisclosed.

Response: Respectfully, we disagree with the statement that "it is not clear what the various tests are reflecting (tinnitus or hyperacusis)." We believe that this is overstating the perceived flaws in acoustic startle reflex gap-detection testing. Certainly, like any behavioral test for tinnitus, gap-detection has its limitaton, and variability in startle responsivity is one of them. Also, an increase in startle amplitude (often resulting from factors that could cause tinnitus) is often interpreted as hyperacusis-like behavior (Pace and Zhang 2013; Sun et al., 2012; Chen et al, 2013; Ison et al, 2007). However, gap-detection has been successfully correlated with operant conditioning behavioral models (Turner et al., 2006) and has behaviorally indicated tinnitus in rats that exhibit the expected correlates of tinnitus (Zhang et al, 2011; Llano et al., 2013; Middleton et al., 2011). Therefore, stating that acoustic startle reflex gap-detection testing is summarily unclear is an overstatement and should be avoided. It is also beyond the scope and intent of this paper to discuss the merits of different behavioral tests for tinnitus.

Question 9: Startle force might be reduced not only to acoustical startle – tactile startle or other controls are necessary.

Response: While blast exposure may influence polysensory startle reactivity, we believe that investigating this is not integral to interpreting our results. Startle force was not reduced in the Untreated group at most time points, indicating that exposure to blast and the effects of blast do not always reduce startle force and that acoustical startle could distinguish this. Since startle force reduction was not seen in all blasted animals at the majority of time points, it is not crucial to test reactivity to other polysensory startle stimuli, although such a study would be interesting.

Question 10: Blast induced TBI that might occur in the rat is not introduced. Some literature is not appropriate. E.g. the studies of Walhausser-Franke and coworkers are on Mongolian gerbils, after Na-Salicylate or noise exposure but never tested for tinnitus. To my knowledge, the only study testing tinnitus in Gerbils is from Nowotny et al. JASA 2011. For the upregulation of NO by Sildenafil, a citation is needed. Thakur et al. 2013 report neuroprotection, not impairment. Literature written in Chinese language is not accessible to everyone. Citation of Pace 2013 should be Pace and Zhang 2013…. Citation Thaktur et al 2013 is ambiguously used.

Response: The Walhausser-Franke study reported c-fos upregulation as an indication of increased activity in the auditory cortex and dorsal cochlear nucleus, which is relevant in our discussion of the potential neural generators of tinnitus. Although they did not test their animals for tinnitus, they euthanized their gerbils no later than 3 hours after salicylate injection or 7 hours after noise exposure. The robustness of acute tinnitus following induction procedures has been well-established (Kraus et al., 2010, Norman et al., 2012, Pace and Zhang, 2013). Furthermore, Walhausser-Franke and colleagues compared their salicylate treated and noise-exposed animals and showed similar molecular biomarkers (c-fos upregulation), further supporting the notion that animals sustained tinnitus. In addition to Nowotny et al. JASA 2011, another investigation did test gerbils for behavioral evidence of tinnitus (Ahlf et al 2012).

The following citation is added to our introduction:
Increased NO expression has been observed in cardiomyocytes after treatment with sildenafil (Prabhu 2013).
Thakur 2013 discusses a case study in which most patients were not affected by tadalafil, but 3 patients showed possible evidence of increased hearing thresholds, which would indicate hearing impairment, not protection. The Chinese study that was cited has an English abstract that is accessible on PubMed. The scientific information from this article should not be ignored due to language differences.
We have changed "Pace 2013" to "Pace and Zhang 2013".

Question 11: The noise burst startle is not defined.

Response: We had previously described the noise burst startle as a 50 ms, 115 dB SPL noise burst. We have now added that it is a "white noise."

Question 12: The author's used 10-ms clicks for acoustic stimuli in the ABRs. This is unusual and needs explanation.

Response: The 10 ms tone duration that we used is longer than that of several other studies, where the duration is typically around 5 ms. Nevertheless, others have also used tone durations near 10 ms (Dehmel et al., 2012; Church, et al., 2013), and the Church lab in particular is well-established in ABR methodology. Therefore, we feel that using 10 ms tones is not problematic.

Question 13: The paradigm of a silent gap reducing the response to the startle response because of tinnitus only works if the hearing of the animals is not changed by the treatment. Otherwise a loss of loudness discrimination leads to the same result and will be interpreted as tinnitus. Since the authors demonstrate that also the detectability of a prepulse for inhibition of startle response (PPI) is changed, the preconditions are not achieved.

Response: Respectfully, we disagree with the reviewer. First, it has been shown that unilateral hearing threshold shifts, even when permanent, do not always alter gap-detection (cite Turner et al., 2006; Pace and Zhang 2013, and others). Second, although PPI detectability was in fact worsened following blast exposure, especially at 1 week post-blast, this does not account for all behavioral evidence of tinnitus. The untreated group, for instance, demonstrated no deficits in 26-28 kHz PPI from 4 weeks post-blast onward while simultaneously exhibiting 26-28 kHz tinnitus, indicating that tinnitus was not due to compromised hearing detection.

Question 14: SPL of prepulse SPL and background noise are not given.

Response: This information was given in the Methods section, under the second paragraph of the section entitled "Gap-detection and prepulse inhibition testing (before blast exposure)"

Question 15: Onset behavioral evidence for tinnitus is unclear.

Response: It is true that startle amplitude was significantly reduced in both blasted groups (Treated and Untreated) and that, combined with PPI deficits and ABR threshold shifts, this raises some concerns regarding the validity of behavioral manifestation of tinnitus. Nevertheless, startle amplitude remained significantly elevated compared to the noise floor amplitude, indicating that startle responsivity was still intact and that it still bore the capacity to be suppressed. Additionally, it has been well-established that tinnitus occurs immediately after acoustic trauma (Kraus et al., 2010, Norman et al., 2012, Pace and Zhang, 2013), which lends further support to onset behavioral evidence of tinnitus. We added a statement regarding this in the Discussion section.

Question 16: The surface righting latency would be dependent on vestibular function. How is this impaired by the blast? …Sildenafil might reduce inflammatory reactions and increase perfusion. After disruption of the blood brain barrier this effect is very important. But the brain injury is not necessarily the cause for surface righting latency: A vestibular defect can not be excluded from the authors.

Response: Vestibular symptoms related to blast injury include spatial perception and navigation deficits, in addition to dizziness and disequilibrium (Frake 2012). In our experience, the animals were completely unconscious for a majority of the time before they regained consciousness and righted

themselves. Nevertheless, we acknowledge that a vestibular defect cannot be completely excluded and have made a statement regarding vestibular effects in our discussion.

Question 17: Less hearing impairment on some measurements. This is not a precise result.

Response: We wrote “less hearing impairment” since sildenafil treatment exerted complex effects on blast-induced hearing impairment, and we were trying to succinctly summarize our results before moving onto specifics. We explained what “less hearing impairment” entails in the body of the Results section.

Question 18: This may implicate complicated effects. hand-waving for discussion, not for results.

Response: Before going into detailed discussions, we tend to give an overview of our results and their potential implications.

Question 19: The untreated group showed significantly less reduction in startle force. Can this be an unspecific effect of sildenafil?

Response: The untreated group did not receive sildenafil.

Question 20: By 1 week post-blast, significant threshold shifts. From the figure, thresholds for all frequencies are still increased.

Response: We already stated in the manuscript that threshold shifts were significant.

Question 21: In the current study, strong tinnitus. may have negated the effect of any reduction in hearing impairment on behavioral performance. This is not the correct interpretation. The authors show changes in behavior and changes in hearing threshold, so they can not speculate that hearing loss will not influence behavior.

Response: We were explaining one of the more complicated findings of this study, which is a little difficult to articulate. Specifically, while the Treated and Untreated groups both exhibited significant hearing loss, the Treated group exhibited overall lower threshold shifts in the right and left ear at 1 day and week post-blast, respectively, as well as less impaired PPI compared to the Untreated group at 1 week post-blast. In spite of this, the Treated group still exhibited robust behavioral evidence of tinnitus at 1 week post-blast, indicating that their reduced hearing impairment did not diminish tinnitus development. We have addressed the effects of hearing thresholds and hearing detection in our previous responses.

Question 22: Parts of the discussion are highly speculative, particularly how plastic changes might be suppressed by Sildenafil treatment. The authors do not give mechanistic evidence for this.

Response: We accept the reviewer’s advice and have adjusted the text as such:

These plastic changes may have been further modulated by sildenafil treatment during the third week post-blast, but were allowed to take place by post-blast week 7 when high-frequency tinnitus reemerged.

We have addressed the proposed mechanistic influence of sildenafil in our previous responses.

Question 23: sildenafil facilitates blast-induced reduction on the startle force. At beginning of week 1 the blast-exposed groups have similarly reduced startle force!

Response: At the beginning of the paragraph where we stated "…sildenafil facilitates blast-induced reduction of the startle force," we also stated that both groups exhibited an initial decrease in startle force, meaning at 1 week post-blast. Furthermore, the quoted text is simply part of a statement describing how the data initially appears (from post-blast 3 weeks onward, the Treated group actually exhibits lower startle force compared to the Untreated group). We later say that upon careful examination, sildenafil itself may not be the factor(s) reducing startle force. We explain this later in the discussion as a combination of hyperacusis amelioration and TBI effects.

Question 24: Citation Angrilli et al 2008: These authors report reduced startle stimulus unpleasantness, contradictory to the concept of hyperacusis that the authors use to interpret their data. Any explanation?

Response: In Angrilli et al., 2008, the authors reported reduced acoustic startle responsivity as a result of orbitofrontal cortex lesions sustained from car or ski accidents. The reason we cited this paper was to mention that TBI can lead to reduced acoustic startle responsivity. This fact is important since TBI reduction of the startle amplitude and hyperacusis elevation of the startle amplitude is the best explanation for the observed behavioral changes in the Treated and Untreated groups. This coincides with the apparent sildenafil-induced attenuation of tinnitus and hyperacusis. Furthermore, while Angrilli et al., 2008 studied patients with strictly blunt-trauma TBI, our study employed a blast exposure that included both blunt and auditory trauma. The subjects in our study developed tinnitus and hearing loss, both of which are highly correlated with hyperacusis, which is a key distinction compared to Angrilli et al., 2008.

Question 25: blast induced neuroplasticity (Mao et al., 2012). The cited study is not containing data that evidence blast-induced neuroplasticity. The change in axial diffusivity from the imaging is only proposing neuroplasticity.

Response: We have taken the reviewer's advice and have changed "showing blast-induced neuroplasticity" to "suggesting blast-induced neuroplasticity." Nevertheless, DTI is becoming an increasingly accepted and validated neuroimaging tool, so we feel confident that it can reliably indicate neuroplasticity.

Question 26: The point by point explanation in the figure captions should give an overview of the data. Furthermore, interpretations of suppression, recovery, reemerging, returning, witness, etc., are speculative interpretations:

Response: We have taken the reviewer's recommendations and have adjusted the figure legends accordingly.

Question 27: Figure 2 is indicating significant surface righting latency for treated groups already after second blast. Groups may not have been homogenously distributed. I do not understand how the sham-exposed group has longer latencies from the very beginning but I guess blasts for different groups did not take place at the same day and this might increase variability.

Response: Due to the nature of our experimental design, we were unable to perform all blast procedures in one day. Thus, a certain degree of variability is expected. Although shams did have slightly longer initial surface righting latency, it was not statistically significant. We were much more

interested in the cumulative effects of multiple blasts, wherein we demonstrated a clear dose-dependent effect on surfacing righting latency. Both blasted groups also had similar surface righting latencies following the third blast exposure, indicating that the three blasts had similar cumulative effects on Treated and Untreated rats.

Question 28: Figure 4: why does the sham group suddenly have reduced startle force over all frequencies? Is there an explanation for this? Is this an unspecific Sildenafil effect?

Response: Startle force is known to fluctuate to some degree across testing sessions and under different circumstances. For example, the animals were all transported between two buildings and this could very well have caused some variation in startle force, even for the Sham group. We made every possible effort to create very similar experiences for our three groups to take these variations into account. We have added a statement regarding this in the Discussion section.

Question 29: PDE-5 is not the same as a PDE-5 inhibitor
Response: We corrected this.

References

Franke LM, Walker WC, Cifu DX, Ochs AL, Lew HL. Sensorintegrative dysfunction underlying vestibular disorders after traumatic brain injury: a review. J Rehabil Res Dev. 2012;49(7):985-94.

Prabhu LV, Rai R, Ranade AV, Kini H, Krishnamurthy A, Bernhardt KL. Effect of sildenafil-induced nitric oxide on the histomorphology of cardiomyocytes in male rats. Int J Appl Basic Med Res. 2013;3(2):84-7.

Stegbauer J, Friedrich S, Potthoff SA, et al. Phosphodiesterase 5 Attenuates the Vasodilatory Response in Renovascular Hypertension. PLoS ONE. 2013;8(11):e80674.

Wang L, Chopp M, Szalad A, et al. Phosphodiesterase-5 is a therapeutic target for peripheral neuropathy in diabetic mice. Neuroscience. 2011;193:399-410.

Page AJ, O'donnell TA, Cooper NJ, Young RL, Blackshaw LA. Nitric oxide as an endogenous peripheral modulator of visceral sensory neuronal function. J Neurosci. 2009;29(22):7246-55.

Ahlf S, Tziridis K, Korn S, Strohmeyer I, Schulze H. Predisposition for and prevention of subjective tinnitus development. PLoS ONE. 2012;7(10):e44519.

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, et al. (2006) Gap detection deficits in rats with tinnitus: a potential novel screening tool. BehavNeurosci 120: 188-195.

Chen G, Lee C, Sandridge SA, Butler HM, Manzoor NF, et al. (2013) Behavioral Evidence for Possible Simultaneous Induction of Hyperacusis and Tinnitus Following Intense Sound Exposure. J Assoc Res Otolaryngol.

Sun W, Deng A, Jayaram A, Gibson B (2012) Noise exposure enhances auditory cortex responses related to hyperacusis behavior. Brain Res 1485: 108-116.

Pace E, Zhang J.S. (2013) Noise-Induced Tinnitus Using Individualized Gap Detection Analysis and Its Relationship with Hyperacusis, Anxiety, and Spatial Cognition. PLoS ONE 8:e75011.

Ison JR, Allen PD, O'Neill WE (2007) Age-related hearing loss in C57BL/6J mice has both frequency-specific and non-frequency-specific components that produce a hyperacusis-like exaggeration of the acoustic startle reflex. JAssocResOtolaryngol 8: 539-550.

Zhang J, Zhang Y, Zhang X (2011) Auditory cortex electrical stimulation suppresses tinnitus in rats. JAssocResOtolaryngol 12: 185-201.

Llano DA, Turner J, Caspary DM. Diminished cortical inhibition in an aging mouse model of chronic tinnitus.J Neurosci. 2012 Nov 14;32(46):16141-8.

Middleton JW, Kiritani T, Pedersen C, Turner JG, Shepherd GM, Tzounopoulos T. Mice with behavioral evidence of tinnitus exhibit dorsal cochlear nucleus hyperactivity because of decreased GABAergic inhibition. Proc Natl Acad Sci U S A. 2011 May 3;108(18):7601-6.

Norman M, Tomscha K, Wehr M (2012) Isoflurane blocks temporary tinnitus. Hear Res 290:64-71.

Kraus KS, Mitra S, Jimenez Z, Hinduja S, Ding D, Jiang H, Gray L, Lobarinas E, Sun W, Salvi RJ (2010) Noise trauma impairs neurogenesis in the rat hippocampus. Neuroscience 167:1216-1226.

Church MW, Zhang JS, Langford MM, Perrine SA (2013). 'Ecstasy' enhances noise-induced hearing loss. Hearing Research. Aug;302-92-106.

Dehmel S, Pradhan S, Koehler S, Bledsoe S, Shore S (2012) Noise overexposure alters long-term somatosensory-auditory processing in the dorsal cochlear nucleus--possible basis for tinnitus-related hyperactivity? J Neurosci 32: 1660-1671.

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December 17, 2013

Dear Drs. Hirsch and Knipper,

We would greatly appreciate your consideration of our REVISED manuscript entitled "Therapeutic Effect of Sildenafil on Blast-Induced Tinnitus and Auditory Impairment" for publication in Neuroscience. We have addressed the comments of reviewers.

**This manuscript is in accordance with the authorship statement of ethical standards for manuscripts submitted to Neuroscience.**

Thank you very much again for your time and consideration.

Sincerely,

Jinsheng Zhang, Ph.D.
Professor
Associate Chair for Research

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Response: The experimental design of our investigation focuses mainly on behavioral evidence of tinnitus and hearing loss, and is our initial report. Histological analysis is beyond the scope of this paper and would be more appropriate in a separate, independent discussion. However, we added a statement in the Discussion recommending future histological analysis. We would also like to note that we have discussed other proposed mechanisms in our paper, including enhanced neurogenesis, activation of the Akt pathway, and upregulation of endothelial nitric oxide synthase, which has been shown to enhance perfusion.

Question 5: Furthermore, some weaknesses limit the acceptation of the data. The Sildenafil application was for a first week then there was one week interruption, then another week of treatment. It is not clear why this treatment regime was chosen. This complicates the interpretability of the data.

Response: One full week of treatment was originally our intention, but after seeing a mild therapeutic effect during the second week (washout), we decided to give another week-long dose of the drug to achieve a more robust therapeutic effect. We also wanted to avoid continuously administering sildenafil for too long a period of time, as some report has indicated that PDE5-inhibitors can contribute to hearing loss. We have now added these statements in the Methods sections to clarify. In spite of these aforementioned factors, they do not, in any way, negate the observation that sildenafil provides protection from blast induced auditory damage. Now that we have established the therapeutic potential of sildenafil, we can attempt to identify an optimal treatment regimen, although this should be done in a separate study and discussion that compares different treatment strategies.

Question 6: The statistical presentation is very awkward. Even if the authors used this kind of presentation in previous publications, I doubt it is correct. If 1-way-ANOVA was chosen (this information is not given in the methods) only one single F-value can result from a single group comparison, followed by indication of significance from the pair wise comparison by the Bonferroni post-hoc test. In contrast to this, the authors give an F-value for every single frequency and time point. This may be not correct. If 2-way-ANOVA or 3-way ANOVA had been used (unclear whether it was) the authors must tell which factors / source of variation they refer to when they give the F-value from the ANOVA. Additionally, the time series data are not independent (same animals) and need adjustment for multiple testing by correction of family

wise error and/or modeling of multivariant ANOVA (MANOVA). The statistics must be explained.

Response: To clarify our statistical analysis, we indicated that we used one-way ANOVA and post-hoc Bonferroni in the Data Analysis section. In this study, we compared the degree of change (gap/PPI percent change, startle amplitude change, and threshold shifts) between groups at each time point. We did not compare data from different time points within the same group – this type of analysis would not be useful since tinnitus, hearing loss, hyperacusis, and TBI-related effects could themselves change over the course of time and confound sildenafil-treatment effects. If we had compared data across time from within the same group, then yes, we would only have one F value for between group comparisons. However, since we compared between the three groups at each time point, we had an F value for the overall three-group comparison, and post-hoc values for specific between group comparisons.

Question 7: The authors plus one ear to maintain hearing on one ear after blast. I guess this was done since the startle model is not giving reliable and reproducible results if the animals suffer from a general loss of auditory thresholds (this is the precondition the authors explain in their methods part). However, a hearing loss remains on both ears, also on the plugged, desired protected ear. It is unclear how this changes the startle responses (those are then interpreted as signs for tinnitus and hyperacusis).

Response: Although both ears sustained hearing loss through one week post-blast, only the exposed left ear retained significant thresholds shifts beyond this time point. It has been shown that unilateral hearing threshold shifts, even when permanent, do not necessarily alter detectability of the gap or startle responsivity (Turner et al., 2006; Pace and Zhang 2013; Sun et al., 2012; Chen et al, 2013).

Question 8: Startle responses are recently carefully revisited as models for 'hyperacusis', since it is not clear what the various tests are reflecting (tinnitus or hyperacusis, see e.g. JJ Eggermont Hearing Research 2013). The authors themselves agree that their results from the startle experiments are complex and complicated. For a general readership, these results are undisclosed.

Response: Respectfully, we disagree with the statement that “it is not clear what the various tests are reflecting (tinnitus or hyperacusis).” We believe that this is overstating the perceived flaws in acoustic startle reflex gap-detection testing. Certainly, like any behavioral test for tinnitus, gap-detection has its limitaton, and variability in startle responsivity is one of them. Also, an increase in startle amplitude (often resulting from factors that could cause tinnitus) is often interpreted as hyperacusis-like behavior (Pace and Zhang 2013; Sun et al., 2012; Chen et al, 2013; Ison et al, 2007). However, gap-detection has been successfully correlated with operant conditioning behavioral models (Turner et al., 2006) and has behaviorally indicated tinnitus in rats that exhibit the expected correlates of tinnitus (Zhang et al, 2011; Llano et al., 2013; Middleton et al., 2011). Therefore, stating that acoustic startle reflex gap-detection testing is summarily unclear is an overstatement and should be avoided. It is also beyond the scope and intent of this paper to discuss the merits of different behavioral tests for tinnitus.

Question 9: Startle force might be reduced not only to acoustical startle – tactile startle or other controls are necessary.

Response: While blast exposure may influence polysensory startle reactivity, we believe that investigating this is not integral to interpreting our results. Startle force was not reduced in the Untreated group at most time points, indicating that exposure to blast and the effects of blast do not always reduce startle force and that acoustical startle could distinguish this. Since startle force reduction was not seen in all blasted animals at the majority of time points, it is not crucial to test reactivity to other polysensory startle stimuli, although such a study would be interesting.

Question 10: Blast induced TBI that might occur in the rat is not introduced. Some literature is not appropriate. E.g. the studies of Walhausser-Franke and coworkers are on Mongolian gerbils, after Na-Salicylate or noise exposure but never tested for tinnitus. To my knowledge, the only study testing tinnitus in Gerbils is from Nowotny et al. JASA 2011. For the upregulation of NO by Sildenafil, a citation is needed. Thakur et al. 2013 report neuroprotection, not impairment. Literature written in Chinese language is not accessible to everyone. Citation of Pace 2013 should be Pace and Zhang 2013…. Citation Thaktur et al 2013 is ambiguously used.

Response: The Walhausser-Franke study reported c-fos upregulation as an indication of increased activity in the auditory cortex and dorsal cochlear nucleus, which is relevant in our discussion of the potential neural generators of tinnitus. Although they did not test their animals for tinnitus, they euthanized their gerbils no later than 3 hours after salicylate injection or 7 hours after noise exposure. The robustness of acute tinnitus following induction procedures has been well-established (Kraus et al., 2010, Norman et al., 2012, Pace and Zhang, 2013). Furthermore, Walhausser-Franke and colleagues compared their salicylate treated and noise-exposed animals and showed similar molecular biomarkers (c-fos upregulation), further supporting the notion that animals sustained tinnitus. In addition to Nowotny et al. JASA 2011, another investigation did test gerbils for behavioral evidence of tinnitus (Ahlf et al 2012).

The following citation is added to our introduction:
Increased NO expression has been observed in cardiomyocytes after treatment with sildenafil (Prabhu 2013).
Thakur 2013 discusses a case study in which most patients were not affected by tadalafil, but 3 patients showed possible evidence of increased hearing thresholds, which would indicate hearing impairment, not protection. The Chinese study that was cited has an English abstract that is accessible on PubMed. The scientific information from this article should not be ignored due to language differences.
We have changed “Pace 2013” to “Pace and Zhang 2013”.

Question 11: The noise burst startle is not defined.

Response: We had previously described the noise burst startle as a 50 ms, 115 dB SPL noise burst. We have now added that it is a “white noise.”

Question 12: The author‟s used 10-ms clicks for acoustic stimuli in the ABRs. This is unusual and needs explanation.

Response: The 10 ms tone duration that we used is longer than that of several other studies, where the duration is typically around 5 ms. Nevertheless, others have also used tone durations near 10 ms (Dehmel et al., 2012; Church, et al., 2013), and the Church lab in particular is well-established in ABR methodology. Therefore, we feel that using 10 ms tones is not problematic.

Question 13: The paradigm of a silent gap reducing the response to the startle response because of tinnitus only works if the hearing of the animals is not changed by the treatment. Otherwise a loss of loudness discrimination leads to the same result and will be interpreted as tinnitus. Since the authors demonstrate that also the detectability of a prepulse for inhibition of startle response (PPI) is changed, the preconditions are not achieved.

Response: Respectfully, we disagree with the reviewer. First, it has been shown that unilateral hearing threshold shifts, even when permanent, do not always alter gap-detection (cite Turner et al., 2006; Pace and Zhang 2013, and others). Second, although PPI detectability was in fact worsened following blast exposure, especially at 1 week post-blast, this does not account for all behavioral evidence of tinnitus. The untreated group, for instance, demonstrated no deficits in 26-28 kHz PPI from 4 weeks post-blast onward while simultaneously exhibiting 26-28 kHz tinnitus, indicating that tinnitus was not due to compromised hearing detection.

Question 14: SPL of prepulse SPL and background noise are not given.

Response: This information was given in the Methods section, under the second paragraph of the section entitled "*Gap-detection and prepulse inhibition testing (before blast exposure)"*

Question 15: Onset behavioral evidence for tinnitus is unclear.

Response: It is true that startle amplitude was significantly reduced in both blasted groups (Treated and Untreated) and that, combined with PPI deficits and ABR threshold shifts, this raises some concerns regarding the validity of behavioral manifestation of tinnitus. Nevertheless, startle amplitude remained significantly elevated compared to the noise floor amplitude, indicating that startle responsivity was still intact and that it still bore the capacity to be suppressed. Additionally, it has been well-established that tinnitus occurs immediately after acoustic trauma (Kraus et al., 2010, Norman et al., 2012, Pace and Zhang, 2013), which lends further support to onset behavioral evidence of tinnitus. We added a statement regarding this in the Discussion section.

Question 16: The surface righting latency would be dependent on vestibular function. How is this impaired by the blast? …Sildenafil might reduce inflammatory reactions and increase perfusion.
After disruption of the blood brain barrier this effect is very important. But the brain injury is not necessarily the cause for surface righting latency: A vestibular defect can not be excluded from the authors.

Response: Vestibular symptoms related to blast injury include spatial perception and navigation deficits, in addition to dizziness and disequilibrium (Frake 2012). In our experience, the animals

were completely unconscious for a majority of the time before they regained consciousness and righted themselves. Nevertheless, we acknowledge that a vestibular defect cannot be completely excluded and have made a statement regarding vestibular effects in our discussion.

Question 17: Less hearing impairment on some measurements. This is not a precise result.

Response: We wrote “less hearing impairment” since sildenafil treatment exerted complex effects on blast-induced hearing impairment, and we were trying to succinctly summarize our results before moving onto specifics. We explained what “less hearing impairment” entails in the body of the Results section.

Question 18: This may implicate complicated effects. hand-waving for discussion, not for results.

Response: Before going into detailed discussions, we tend to give an overview of our results and their potential implications.

Question 19: The untreated group showed significantly less reduction in startle force. Can this be an unspecific effect of sildenafil?

Response: The untreated group did not receive sildenafil.

Question 20: By 1 week post-blast, significant threshold shifts. From the figure, thresholds for all frequencies are still increased.

Response: We already stated in the manuscript that threshold shifts were significant.

Question 21: In the current study, strong tinnitus. may have negated the effect of any reduction in hearing impairment on behavioral performance. This is not the correct interpretation. The authors show changes in behavior and changes in hearing threshold, so they can not speculate that hearing loss will not influence behavior.

Response: We were explaining one of the more complicated findings of this study, which is a little difficult to articulate. Specifically, while the Treated and Untreated groups both exhibited significant hearing loss, the Treated group exhibited overall lower threshold shifts in the right and left ear at 1 day and week post-blast, respectively, as well as less impaired PPI compared to the Untreated group at 1 week post-blast. In spite of this, the Treated group still exhibited robust behavioral evidence of tinnitus at 1 week post-blast, indicating that their reduced hearing impairment did not diminish tinnitus development. We have addressed the effects of hearing thresholds and hearing detection in our previous responses.

Question 22: Parts of the discussion are highly speculative, particularly how plastic changes might be suppressed by Sildenafil treatment. The authors do not give mechanistic evidence for this.

Response: We accept the reviewer‟s advice and have adjusted the text as such:

These plastic changes may have been further modulated by sildenafil treatment during the third week post-blast, but were allowed to take place by post-blast week 7 when high-frequency tinnitus reemerged.

We have addressed the proposed mechanistic influence of sildenafil in our previous responses.

Question 23: sildenafil facilitates blast-induced reduction on the startle force. At beginning of week 1 the blast-exposed groups have similarly reduced startle force!

Response: At the beginning of the paragraph where we stated "…sildenafil facilitates blast-induced reduction of the startle force," we also stated that both groups exhibited an *initial* decrease in startle force, meaning at 1 week post-blast. Furthermore, the quoted text is simply part of a statement describing how the data initially appears (from post-blast 3 weeks onward, the Treated group actually exhibits lower startle force compared to the Untreated group). We later say that upon careful examination, sildenafil itself may not be the factor(s) reducing startle force. We explain this later in the discussion as a combination of hyperacusis amelioration and TBI effects.

Question 24: Citation Angrilli et al 2008: These authors report reduced startle stimulus unpleasantness, contradictory to the concept of hyperacusis that the authors use to interpret their data. Any explanation?

Response: In Angrilli et al., 2008, the authors reported reduced acoustic startle responsivity as a result of orbitofrontal cortex lesions sustained from car or ski accidents. The reason we cited this paper was to mention that TBI can lead to reduced acoustic startle responsivity. This fact is important since TBI reduction of the startle amplitude and hyperacusis elevation of the startle amplitude is the best explanation for the observed behavioral changes in the Treated and Untreated groups. This coincides with the apparent sildenafil-induced attenuation of tinnitus and hyperacusis. Furthermore, while Angrilli et al., 2008 studied patients with strictly blunt-trauma TBI, our study employed a blast exposure that included both blunt and auditory trauma. The subjects in our study developed tinnitus and hearing loss, both of which are highly correlated with hyperacusis, which is a key distinction compared to Angrilli et al., 2008.

Question 25: blast induced neuroplasticity (Mao et al., 2012). The cited study is not containing data that evidence blast-induced neuroplasticity. The change in axial diffusivity from the imaging is only proposing neuroplasticity.

Response: We have taken the reviewer‟s advice and have changed "showing blast-induced neuroplasticity" to "suggesting blast-induced neuroplasticity." Nevertheless, DTI is becoming an

increasingly accepted and validated neuroimaging tool, so we feel confident that it can reliably indicate neuroplasticity.

Question 26: The point by point explanation in the figure captions should give an overview of the data. Furthermore, interpretations of suppression, recovery, reemerging, returning, witness, etc., are speculative interpretations:

Response: We have taken the reviewer‟s recommendations and have adjusted the figure legends accordingly.

Question 27: Figure 2 is indicating significant surface righting latency for treated groups already after second blast. Groups may not have been homogenously distributed. I do not understand how the sham-exposed group has longer latencies from the very beginning but I guess blasts for different groups did not take place at the same day and this might increase variability.

Response: Due to the nature of our experimental design, we were unable to perform all blast procedures in one day. Thus, a certain degree of variability is expected. Although shams did have slightly longer initial surface righting latency, it was not statistically significant. We were much more interested in the cumulative effects of multiple blasts, wherein we demonstrated a clear dose-dependent effect on surfacing righting latency. Both blasted groups also had similar surface righting latencies following the third blast exposure, indicating that the three blasts had similar cumulative effects on Treated and Untreated rats.

Question 28: Figure 4: why does the sham group suddenly have reduced startle force over all frequencies? Is there an explanation for this? Is this an unspecific Sildenafil effect?

Response: Startle force is known to fluctuate to some degree across testing sessions and under different circumstances. For example, the animals were all transported between two buildings and this could very well have caused some variation in startle force, even for the Sham group. We made every possible effort to create very similar experiences for our three groups to take these variations into account. We have added a statement regarding this in the Discussion section.

Question 29: PDE-5 is not the same as a PDE-5 inhibitor
Response: We corrected this.

References

Franke LM, Walker WC, Cifu DX, Ochs AL, Lew HL. Sensorintegrative dysfunction underlying vestibular disorders after traumatic brain injury: a review. J Rehabil Res Dev. 2012;49(7):985-94.

Prabhu LV, Rai R, Ranade AV, Kini H, Krishnamurthy A, Bernhardt KL. Effect of sildenafil-induced nitric oxide on the histomorphology of cardiomyocytes in male rats. Int J Appl Basic Med Res. 2013;3(2):84-7.

Stegbauer J, Friedrich S, Potthoff SA, et al. Phosphodiesterase 5 Attenuates the Vasodilatory Response in Renovascular Hypertension. PLoS ONE. 2013;8(11):e80674.

Wang L, Chopp M, Szalad A, et al. Phosphodiesterase-5 is a therapeutic target for peripheral neuropathy in diabetic mice. Neuroscience. 2011;193:399-410.

Page AJ, O'donnell TA, Cooper NJ, Young RL, Blackshaw LA. Nitric oxide as an endogenous peripheral modulator of visceral sensory neuronal function. J Neurosci. 2009;29(22):7246-55.

Ahlf S, Tziridis K, Korn S, Strohmeyer I, Schulze H. Predisposition for and prevention of subjective tinnitus development. PLoS ONE. 2012;7(10):e44519.

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, et al. (2006) Gap detection deficits in rats with tinnitus: a potential novel screening tool. BehavNeurosci 120: 188-195.

Chen G, Lee C, Sandridge SA, Butler HM, Manzoor NF, et al. (2013) Behavioral Evidence for Possible Simultaneous Induction of Hyperacusis and Tinnitus Following Intense Sound Exposure. J Assoc Res Otolaryngol.

Sun W, Deng A, Jayaram A, Gibson B (2012) Noise exposure enhances auditory cortex responses related to hyperacusis behavior. Brain Res 1485: 108-116.

Pace E, Zhang J.S. (2013) Noise-Induced Tinnitus Using Individualized Gap Detection Analysis and Its Relationship with Hyperacusis, Anxiety, and Spatial Cognition. PLoS ONE 8:e75011.

Ison JR, Allen PD, O'Neill WE (2007) Age-related hearing loss in C57BL/6J mice has both frequency-specific and non-frequency-specific components that produce a hyperacusis-like exaggeration of the acoustic startle reflex. JAssocResOtolaryngol 8: 539-550.

Zhang J, Zhang Y, Zhang X (2011) Auditory cortex electrical stimulation suppresses tinnitus in rats. JAssocResOtolaryngol 12: 185-201.

Llano DA, Turner J, Caspary DM. Diminished cortical inhibition in an aging mouse model of chronic tinnitus.J Neurosci. 2012 Nov 14;32(46):16141-8.

Middleton JW, Kiritani T, Pedersen C, Turner JG, Shepherd GM, Tzounopoulos T. Mice with behavioral evidence of tinnitus exhibit dorsal cochlear nucleus hyperactivity because of decreased GABAergic inhibition. Proc Natl Acad Sci U S A. 2011 May 3;108(18):7601-6.

Norman M, Tomscha K, Wehr M (2012) Isoflurane blocks temporary tinnitus. Hear Res 290:64-71.

Kraus KS, Mitra S, Jimenez Z, Hinduja S, Ding D, Jiang H, Gray L, Lobarinas E, Sun W, Salvi RJ (2010) Noise trauma impairs neurogenesis in the rat hippocampus. Neuroscience 167:1216-1226.

Church MW, Zhang JS, Langford MM, Perrine SA (2013). 'Ecstasy' enhances noise-induced hearing loss. Hearing Research. Aug;302-92-106.

Dehmel S, Pradhan S, Koehler S, Bledsoe S, Shore S (2012) Noise overexposure alters long-term somatosensory-auditory processing in the dorsal cochlear nucleus--possible basis for tinnitus-related hyperactivity? J Neurosci 32: 1660-1671.

## Highlights

- Blast exposure induces behavioral evidence of tinnitus in rats
- Sildenafil generates delayed suppression of high-frequency tinnitus after blast exposure
- Sildenafil improves blast-induced hearing threshold shifts
- Sildenafil does not prevent onset tinnitus and hearing impairment

# Therapeutic Effect of Sildenafil on Blast-Induced Tinnitus and Auditory Impairment

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Running head: Therapeutic effect of sildenafil on blast-induced tinnitus

Number of figures: 5

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## Abbreviations:

| | |
|---|---|
| ABR | auditory brainstem response |
| cGMP | cyclic Guanosine Monophosphate |
| NO | Nitric Oxide |
| PDE-5 | phosphodiesterase type 5 inhibitor |
| PPI | prepulse inhibition |
| TBI | Traumatic Brain Injury |

## Abstract

Blast-induced tinnitus, along with associated auditory impairment and traumatic brain injury, is a primary concern facing military service members. To search for treatment, we investigated the therapeutic effects of sildenafil, a phosphodiesterase-5 inhibitor, given its vasodilation effects and evidence suggesting its beneficial effects on noise-induced hearing loss. Rats were subjected to three consecutive blast exposures at 22 psi and were monitored for tinnitus using a gap-detection acoustic startle reflex paradigm. Hearing thresholds and detection were tested using auditory brainstem responses and prepulse inhibition, respectively. Blasted rats were either treated with sildenafil or tap water following blast exposure, while age-matched sham control rats were treated with sildenafil without blast exposure. Our results showed that sildenafil did not effectively prevent acute tinnitus onset and hearing impairment. Instead, sildenafil significantly suppressed high-frequency tinnitus from 3 to 6 weeks after blast exposure and reduced hearing impairment during the first week after blast exposure. Complex results were observed in the startle force data, where sildenafil-treated rats displayed significantly reduced startle force compared to the untreated control group, suggesting of possible mitigation of traumatic brain injury and suppression of hyperacusis-like percepts. Taken together, sildenafil showed a therapeutic effect on blast-induced tinnitus and audiological impairment in a time-dependent manner. Other regimen such as higher dosage prior to blast exposure and in combination with other treatments deserves investigations to optimize therapeutic effects.

*Key words: Tinnitus, Blast, Traumatic brain injury, Sildenafil (Viagra, Revatio), Hyperacusis, Rat*

## Introduction

Tinnitus, a "ringing" in the ears, is the perception of sound in the absence of external stimuli. It is estimated to affect 50 million Americans, with 30% of affected individuals suffering from severe symptoms (Shargorodsky et al., 2010). Tinnitus sufferers experience a wide range of dysfunction, such as insomnia, difficulty concentrating, and a higher propensity for psychological disorders such as depression and anxiety (Rossiter et al., 2006, Stevens et al., 2007, Crocetti et al., 2009, Hesser and Andersson, 2009, Oishi et al., 2010, Hasson et al., 2011, Hebert et al., 2011, Test et al., 2011). Although many causes of tinnitus are known, including acoustic trauma, hearing impairment, and exposure to drugs like salicylate and quinine (Man and Naggan, 1981, Cahani et al., 1983, Jastreboff et al., 1988, Jastreboff et al., 1991, Hiller and Goebel, 1999, Temmel et al., 1999, Cazals, 2000, Lobarinas et al., 2006), the underlying neuromechanisms of tinnitus remain undetermined and there are no consistent treatment strategies. Due to the growing elderly population and large number of veterans returning from Operations Iraqi Freedom and Enduring Freedom, it is increasingly important to develop effective tinnitus therapies.

Blast exposure from improvised explosive devices and rocket-assisted mortars in overseas war theaters has prompted attention to tinnitus, hearing loss, and traumatic brain injury (TBI) in military service members. Auditory dysfunction is the most common sequela of blast related injuries sustained on the battlefield (Gondusky and Reiter, 2005). Over 62% of blast-injured veterans from Operation Iraq Freedom showed signs of hearing loss and over 38% exhibited tinnitus symptoms (Lew et al., 2007). In 2011, tinnitus was the most common source of disability payments received by veterans (Administration, 2011). In addition to the audiological impact, blast wave exposure can lead to TBI (Hue et al., 2013) and one study found that over

53% of patients recovering from TBI developed tinnitus symptoms (Jury and Flynn 2001). This indicates a correlation between plasticity changes associated with TBI and tinnitus (Lew et al., 2007). Studies on rodent models have corroborated these findings by showing that blast can induce tinnitus (Mao et al., 2012) and hearing threshold shifts (Ewert et al., 2012, Mao et al., 2012, Chen et al., 2013b), increase expression of deafness genes (Valiyaveettil et al., 2012), and alter cochlear blood flow (Chen et al., 2013b), as well as result in TBI-related changes such as blood-brain barrier disruption (Abdul-Muneer et al., 2013, Hue et al., 2013), axonal injury (Long et al., 2009), and increased glial fibrillary acidic protein accumulation (Svetlov et al., 2010, Sajja et al., 2012).

Although the neuropathophysiology of tinnitus remains elusive, it has been suggested that tinnitus occurs due to maladaptive plasticity changes following acoustic trauma (Eggermont and Roberts, 2004). Specifically, injury to the peripheral auditory system, such as damage to the hair cells (Kaltenbach et al., 2002, Bauer et al., 2008) and cochlea (Kujawa and Liberman, 2009), appear to drive central auditory reorganization in the forms of hyperactivity, increased bursting and neural synchrony in structures such as the cochlear nucleus, inferior colliculus, and auditory cortex (Chen and Jastreboff, 1995, Zhang and Kaltenbach, 1998, Kimura and Eggermont, 1999, Seki and Eggermont, 2003, Ma et al., 2006, Eggermont, 2007, Finlayson and Kaltenbach, 2009, Vogler et al., 2011). Additional evidence supports the involvement of increased cerebral blood flow and activity in limbic structures, including the amygdala and hippocampus (Shulman et al., 1995, Wang et al., 2000, Wallhausser-Franke et al., 2003, Zhang et al., 2003, Mahlke and Wallhausser-Franke, 2004). As such, therapeutic interventions, including pharmacological treatment, have been developed to target both the auditory and non-auditory systems. Numerous drugs and compounds have been explored including but not limited to lidocaine (Lenarz, 1986,

Reyes et al., 2002, Baguley et al., 2005), gabapentin (Bauer and Brozoski, 2001, 2006), baclofen (Zheng et al., 2012), and antidepressants (Baldo et al., 2006), however, none have been found to consistently and effectively suppress tinnitus (Langguth and Elgoyhen, 2012).

One unexplored drug that may have the potential to treat tinnitus especially blast-induced tinnitus is sildenafil. Sildenafil is a commercially available phosphodiesterase-5 (PDE-5) inhibitor that is commonly prescribed to treat erectile dysfunction (Boolell, Allen, et al. 1996). Recently, PDE-5 inhibitors like sildenafil have demonstrated neuroprotective potential, such as improvements in cerebral blood flow, reduced apoptotic cell death, and improved functional recovery following ischemic and embolic stroke (Zhang et al., 2002, Zhang et al., 2005, Zhang et al., 2006, Li et al., 2007), and focal cerebral cryolesion (Pifarre, Prado et al. 2010). The key components in the signaling cascade of PDE-5 inhibitors are prevention of cyclic guanosine monophosphate (cGMP) degradation and upregulation of nitric oxide (NO). Increased NO expression, for example, has been observed in cardiomyocytes after sildenafil treatment (Prabhu et al., 2013). Administration of the NO precursor L-arginine within the first 15 minutes of TBI can increase blood flow, NO production, and enhance recovery following TBI using cortical impact (Cherian, Chacko et al. 1999) and fluid-percussion injury models (DeWitt, Smith et al. 1997). Mixed results have been seen in the auditory system, with some studies showing hearing impairment following sildenafil administration (Mukherjee and Shivakumar, 2007, Hong et al., 2008, Maddox et al., 2009, Okuyucu et al., 2009, McGwin, 2010, Snodgrass et al., 2010, Khan et al., 2011, Thakur et al., 2013), while others have shown no effect (Mazurek et al., 2009, Giuliano et al., 2010, Thakur et al., 2013). At the same time, sildenafil treatment has been shown to facilitate recovery of hearing thresholds in noise-exposed guinea pigs (Zhang et al., 2011b) and vardenafil significantly reduced noise-induced hearing loss (Jaumann et al., 2012).

Consequently, the potential for sildenafil to treat blast-induced TBI and its associated tinnitus and hearing loss remains unclear.

In the current study, we assessed the therapeutic effects of sildenafil using our established rat model of blast-induced tinnitus, hearing impairment and related TBI (Mao et al., 2012). Following administration of sildenafil, tinnitus was monitored using gap-detection, and hearing thresholds and auditory detection were assessed using auditory brainstem responses (ABRs) and prepulse inhibition (PPI), respectively. We found that sildenafil suppressed high-frequency tinnitus from 3 to 6 weeks post-blast, after which high-frequency tinnitus reemerged, and that it reduced hearing impairment but only during the first week post-blast. These results suggest that sildenafil can interfere with normative neuroplastic changes following acoustic and blunt force trauma, but on a time- and injury-dependent scale. The possible underlying mechanisms are discussed.

## Materials and methods

*Animal subjects*

Thirty adult Sprague Dawley rats (110 days old, 250-300g) were purchased from Charles River Laboratories (Wilmington, MA). Three animals were initially excluded from the study due to poor startle reflex and two animals died from unrelated causes. Another animal was retrospectively removed from the study due to epistaxis immediately following the first blast and absence of startle responsivity during post-blast testing. Of the remaining 24 animals, 10 were blast-exposed and treated with sildenafil (Treated group), 6 were blast-exposed but were given vehicle tap water (Untreated group), and 8 received sildenafil treatment but no blast exposure (Sham group). A sham-blasted group that did not receive sildenafil treatment was not included as

the Sham-treated group displayed relatively stable behavioral performance and hearing thresholds over time. All procedures were approved by the Institutional Animal Care and Use Committee at Wayne State University and were in accordance with the regulations of the Federal Animal Welfare Act. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

*Gap-detection and prepulse inhibition testing (before blast exposure)*

Animals underwent 8 rounds of behavior testing to stabilize baseline gap-detection and PPI performance prior to blast or Sham-blast exposure. Gap-detection and PPI tests were conducted using acoustic startle reflex hardware and software (Kinder Scientific, Poway, CA), as described elsewhere (Zhang et al., 2011a, Luo et al., 2012, Pace and Zhang, 2013). Briefly, each rat was placed in a custom-built polycarbonate restrainer and set inside a lit startle monitor cabinet equipped with two ceiling speakers for background sound/prepulses and startle stimuli. Restrainers were mounted on top of a platform connected to a piezoelectric transducer, which measured downward startle force. Acoustic stimuli and startle force were calibrated using a Newton impulse calibrator (Kinder Scientific) and a microphone (Model 4016; ACO Pacific, Belmont, CA).

During the gap-detection procedure, rats were exposed to constant 60 dB SPL background noise consisting of 2 kHz bandpass signals from 6-8, 10-12, 14-16, 18-20, or 26-28 kHz, or broadband noise (BBN, 2-30 kHz). They were subjected to either a 50 ms white noise burst startle stimulus (startle only) presented at 115 dB or the startle stimulus preceded by a 40 ms silent period beginning 90 ms before the startle stimulus (GAP). For each testing session, rats were presented 8 times with the startle only and GAP conditions for each frequency bandpass

signal and BBN. The PPI procedure was identical to the gap-detection procedure except that no background sound was presented. Rats were subjected to either the startle stimulus alone (startle only) or the startle stimulus preceded by a 40 ms prepulse beginning 90 ms before the startle stimulus. Prepulses consisted of the same frequencies as those used for background noise.

A two-minute acclimatization period was given at the beginning of each session, followed by two startle stimuli presented without background sound to trigger any initial, exaggerated startle reflexes. Gap-detection and PPI testing sessions lasted a little over thirty minutes each.

*Auditory brainstem response (ABR) recording (before blast exposure)*

ABRs were recorded to evaluate hearing thresholds. Each rat was anesthetized using a mixture of air (0.4 l/min) and isoflurane (2-3%, v/v) and placed in a prone position with its head fixed to a stereotaxic frame. Body temperature was maintained using a warming blanket connected to a thermostatic controller (Harvard Instruments, Holliston, MA). Acoustic stimuli consisted of 10-ms clicks or pure-tone bursts presented at 8, 12, 16, 20, or 28 kHz and delivered through a speaker tube inserted into the external auditory canal. Three subcutaneous platinum-coated tungsten electrodes were used to record ABR waveforms, with the reference electrode inserted below the pinna ipsilateral to the speaker tube, the grounding electrode inserted below the contralateral pinna, and the recording electrode inserted at the vertex. Evoked potentials were bandpass-filtered at 300-3000 Hz, notch-filtered at 60 Hz, and averaged 300-400 times for clicks and tone-bursts, respectively. Data were recorded using BioSigRP® and SigGenRP® software (TDT, Alachua, FL) installed on an IBM computer connected to System 3 TDT workstation.

*Blast exposure*

To induce tinnitus and auditory impairment, rats underwent three consecutive blast exposures. The blast shock waves were generated by a custom-built shock tube located in the Biomedical Engineering Building at Wayne State University (ORA Inc. Fredericksburg, VA). Peak static overpressure of 22 psi was produced with compressed helium and calibrated Mylar sheets (GE Richards Graphics Supplies Inc., Landsville, PA) to produce a free field blast wave. Prior to each exposure, a rat was anesthetized with a mixture of isoflurane (3%) and 0.6 L/min of oxygen for 6 minutes. While anesthetized, the rat was harnessed to a sled and positioned 109 cm inside the open end of the shock tube in a rostro-cephalic orientation towards the oncoming shock waves. The right ear was occluded with an earplug (Mack's®, McKeon Products, Warren, MI) for protection against noise trauma, so that responsivity to acoustic stimuli during gap-detection and PPI testing could be retained.

Immediately after each blast exposure, the still anesthetized rat was placed on its back and monitored for latency to surface right, an indirect marker of unconsciousness (Solt et al., 2011). Surface righting was defined as settling on all four paws. An average of five minutes was given between successful surface righting and anesthesia induction for the subsequent blast exposure, during which time rats were transferred to polycarbonate cages. Immediately after successful surface righting following the last blast exposure, sildenafil was administered to Treated and Sham group rats, while a tap water vehicle was given to the Untreated but blasted group. Sham rats went through the same procedures except that blast exposure was not conducted.

*Sildenafil administration*

Sildenafil tablets (100 mg) were crushed, dissolved in tap water and administered once a day at a 10 mg/kg dosage via oral gavage for 7 days after blast exposure. A longer and continuous treatment regimen was not selected since PDE-5 inhibitors have reportedly contributed to hearing loss (Mukherjee and Shivakumar, 2007, Hong et al., 2008, Maddox et al., 2009, Okuyucu et al., 2009, McGwin, 2010, Snodgrass et al., 2010, Khan et al., 2011, Thakur et al., 2013). Dosage was selected based on previous data (Myers et al., 2011). The Untreated group received similar volume of tap water. Curved, stainless steel, ball-nosed feeding needles (20 ga x 3", Popper and Sons, New Hyde Park, NY) were used to deliver the drug orally and were cleaned with tap water after use. Rats were exposed to the feeding needle several times prior to drug administration to habituate them to the oral gavage procedure and reduce stress. After the initial 7-day round of treatment, rats underwent one week without treatment to assess washout effect. Since our data indicated a mild therapeutic effect, the same treated rats were then given a second 7-day round during the third week post-blast. The pharmacokinetic profile of sildenafil shows detectability in blood plasma within 5 minutes upon oral intake and $T_{max}$ values within 11 minutes (Shin et al., 2006).

*Gap-detection, PPI, and ABR testing (after blast exposure)*

Gap-detection and PPI testing were performed one hour following the last blast exposure and for 8 weeks afterward to track the progression of tinnitus and auditory detection. ABRs were performed for each rat on the day of blast exposure and at 1, 3, and 6 weeks post-blast to monitor recovery of hearing thresholds.

*Data analysis*

Gap-detection data were divided into ratios, as described previously (Zhang et al., 2011a, Luo et al., 2012, Mao et al., 2012, Pace and Zhang, 2013). Briefly, for each frequency or BBN, the response to the GAP condition was divided by the mean response to the associated startle only condition, resulting in a ratio value between 0 and 1. A value close to 0 would indicate strong suppression of the startle reflex in response to silent gaps, and thus healthy status, whereas a value close to 1 would signify little suppression in response to the gap, potentially indicating tinnitus (Turner et al., 2006, Yang et al., 2007, Wang et al., 2009, Kraus et al., 2011, Longenecker and Galazyuk, 2011, Nowotny et al., 2011, Zhang et al., 2011a, Luo et al., 2012, Mao et al., 2012). To determine whether blast exposure had an effect on gap-detection, we subtracted the pre-blast GAP ratio from the post-blast ratio and calculated the percentage of change from pre-blast exposure. This was done for each of the 3 groups for several post-blast time points, including the first and second rounds of sildenafil treatment (1 and 3 weeks post-blast, respectively), and approximately 4, 6 and 7 weeks post-blast. Two to three tests per rat were included in each time point, and the percentage of change was compared between the Treated, Untreated, and Sham groups. A higher percentage of change compared to the Sham group would indicate blast-induced GAP impairment. A lower percentage of change in Treated rats compared to Untreated rats would indicate GAP improvement due to sildenafil treatment. PPI data were analyzed in the same manner, except that PPI ratios were calculated and used instead of GAP ratios, and upward or downward changes in PPI data would indicate auditory detection loss or improvement, respectively.

Recently, it has been shown that a reduction in overall startle force can raise GAP and PPI ratios (Longenecker and Galazyuk, 2011, Lobarinas et al., 2012), potentially leading to false tinnitus diagnoses. To account for this, we subtracted the pre-blast startle force in response to the

startle only condition from post-blast startle force and computed the percentage of startle force change from pre-blast (Pace and Zhang, 2013). This was done for the gap-detection startle only condition (with background noise) and the PPI startle only condition (without background noise) and the change in startle force was compared between Treated, Untreated, and Sham groups.

Surface righting latency was measured after each of the three blasts for each rat and also compared between the three groups. Longer surface righting latencies indicated longer periods of unconsciousness.

Finally, ABR threshold shifts were compared between groups by subtracting the pre-blast threshold from the post-blast threshold. Threshold shifts were determined for each recording time point, including post-blast day 0, and post-blast week 1, 3 and 6. Thresholds were considered to be the lowest sound intensity at which a distinct portion of the biological ABR waveform remained visible.

All between-group comparisons on data from GAP/PPI and ABR testing were conducted using one-way ANOVA with post-hoc Bonferroni to adjust alpha values. Statistical significance was considered when $p < 0.05$.

## Results

Immediately following blast exposure, both the Treated and -Untreated groups exhibited onset behavioral evidence of tinnitus and impaired auditory detection, as well as bilateral hearing threshold shifts at all frequencies. All blasted rats as a whole showed delayed surface to right latency, suggesting that blast exposure contributed to unconsciousness. The Treated group displayed 26-28 kHz tinnitus suppression from 3 to 6 weeks post-blast, after which high-frequency tinnitus reemerged. They also displayed less hearing impairment on some

measurements compared to the Untreated group during the first week post-blast, although this disappeared by the third week. Interestingly, the Untreated group did not exhibit an overall decrease in startle force exhibited by the Treated group and occasionally showed increased startle force, suggesting possible hyperacusis-like precepts (Pace and Zhang, 2013). Taken together, our results indicated that sildenafil suppressed tinnitus and reduced hearing impairment to certain degree, but that this occurred in a time- and injury-dependent fashion.

*Surface righting latency*

Following blast exposures, rats were measured for surface righting latency as an index of unconsciousness. Although there were no statistically significant differences between the Treated, Untreated and Sham groups after any of the three blasts, the Treated and Untreated groups displayed a significantly longer latency for surface righting after their third blast compared to their first (Treated, $p < 0.001$; Untreated, $p = 0.029$; paired t-test), indicating a dose-dependent effect of blast on unconsciousness (Figure 2). The Sham group showed no difference between their first and third post-blast surface righting latencies ($p = 0.103$; paired t-test).

*Gap detection and PPI – ratio change*

Gap-detection and PPI testing were conducted to assess the therapeutic effect of sildenafil on blast-induced tinnitus and auditory detection deficits (Figure 3).

At 1 week post-blast, both the Treated and Untreated groups exhibited significant upward percent change in GAP and PPI ratios, indicative of impairment, at all frequencies compared to the Sham group. Therefore, sildenafil treatment did not prevent immediate blast-induced tinnitus or auditory detection deficits. Compared to the Untreated group, Treated rats showed worse GAP

impairment at 18-20 kHz while the Untreated rats showed worse GAP impairment at 26-28 kHz ($p < 0.05$;Fig. 3A) and worse PPI ratios at 6-12, 18-20, and BBN ($p < 0.05$;Fig. 3B) compared to Treated rats. Greater GAP impairment at 18-20 kHz for the Treated group and 26-28 kHz for the Untreated group implies stronger tinnitus at those frequencies for those groups, respectively. Worse PPI performance at several frequencies in the Untreated group indicated greater overall hearing impairment post-blast, suggesting that while sildenafil treatment exerted no preventative effects, it still significantly reduced auditory detection deficits.

By 3 weeks post-blast, 18-20 kHz tinnitus and robust 26-28 kHz tinnitus persisted in Untreated rats ($p < 0.05$; Fig. 3C), in addition to auditory detection deficits from 10-28 kHz ($p < 0.05$; Fig. 3D). The Treated group demonstrated tinnitus at 6-8 and 18-20 kHz, but showed tinnitus suppression at 26-28 kHz, and auditory detection deficits from 10-28 kHz. Interestingly, although the Treated group demonstrated the worst impairment at 14-16 kHz PPI, this was not associated with worse impairment at 14-16 kHz GAP. This may implicate complicated effects from TBI and/or a difference between gap-detection and PPI neurocircuitry and consequent functioning, the latter of which has been suggested by others (Peiffer et al., 2004, Swetter et al., 2010).

At 4 weeks post-blast, Untreated rats showed tinnitus from 14-28 kHz and BBN, while the Treated group exhibited tinnitus at 18-20 kHz but suppression at all other frequencies ($p < 0.05$; Fig. 3E). The Untreated group also demonstrated auditory detection deficits from 8-20 kHz and BBN, while the Treated group showed deficits from 10-28 kHz ($p < 0.05$; Fig. 3F).

Six weeks following blast exposure, Untreated rats demonstrated tinnitus at 14-16 and 26-28 kHz ($p < 0.05$; Fig. 3G) and an auditory detection deficit at 18-20 kHz ($p < 0.05$; Fig. 3H). Treated rats, meanwhile, exhibited tinnitus from 14-20 kHz and auditory detection deficits from

10-20 kHz. It should be noted that the Untreated group did not show impairment at 10-12 or 14-16 kHz PPI, although these frequencies are impaired at all other time points. This may reflect increased sensitivity to these frequencies during this time point.

Lastly, at 7 weeks post-blast, Untreated rats maintained tinnitus at 26-28 kHz ($p < 0.05$; Fig. 3I) and auditory detection deficits from 6-20 kHz ($p < 0.05$; Fig. 3J), while Treated rats exhibited tinnitus at 6-8 and 26-28 kHz and auditory detection deficits from 6-28 kHz and BBN.

*Gap-detection and prepulse inhibition – startle force change*

Changes in startle force following blast exposure were also monitored, in part to determine whether they could account for changes in ratio values.

Following blast exposure, Treated rats showed significantly decreased startle force in response to the startle only condition with background noise (gap-detection test) and without background noise (PPI test) across almost all time points compared to the Untreated and Sham groups (Figure 4). The exceptions were the 1-week post-blast time point, where Treated and Untreated rats sustained similar reductions in startle force during all background noises ($p = 1.000$; Fig. 4A), and without background noise near all prepulses $p = 1.000$; Fig. 4B) compared to the Sham group. At 6 weeks post-blast, Treated rats showed similar startle force reduction without background noise near all prepulses compared to Sham rats, mostly due to an transient reduction in startle force for the Sham group ($p = 1.000$; Fig. 4H).

Compared to the treated group, the Untreated group showed significantly less reduction in startle force and on occasion displayed increased startle force. The exception was during the first week post-blast, where Untreated rats sustained startle force reduction during all background noises ($p < 0.05$; Fig. 4A) and without background noise near all prepulses ($p <$

0.05; Fig. 4B). Following 1 week post-blast, they only displayed occasional reductions in startle force compared to the Sham group, including during 14-16 kHz at 3 weeks post-blast ($p < 0.05$; Fig. 4C) and at 6 weeks post-blast ($p < 0.05$; Fig. 4G), but showed greater startle force reduction at 7 weeks post-blast during 10-28 kHz background noise ($p < 0.05$; Fig. 4I). They also demonstrated increased startle force without background noise compared to Shams at 3 weeks post-blast near 6-8 kHz ($p < 0.05$; Fig. 4D), at 4 weeks post-blast near 26-28 kHz and BBN ($p < 0.05$; Fig. 4F), at 6 weeks post-blast near all prepulse conditions (Fig. 4H), which was mostly due to reduction in Sham rat startle force, and at 7 weeks post-blast near 26-28 kHz ($p < 0.05$; Fig. 4J). The lack of overall startle force decrease seen in the Untreated group, compared to the Treated group, as well as the occasional increases in startle force may indicate increased startle responsivity. This could in turn implicate the presence of hyperacusis-like precepts and behavior in the Untreated group.

*ABR threshold shifts*

ABR recordings were conducted in the left and right ears to determine if sildenafil had any therapeutic effects on blast-induced threshold shifts. Although we attempted unilateral blast exposure by occluding the right ear with an earplug, the right ears still incurred threshold shifts of over 40 dB (Figure 5). In addition, while there were no significant differences in threshold shifts between the Treated and Untreated groups at individual frequencies, we found an overall reduction in threshold shifts for the Treated group in the right ear at post-blast day 0 ($p < 0.05$; Fig. 5B) and in the left ear at post-blast week 1 ($p < 0.05$; Fig. 5A), suggesting that sildenafil reduced hearing impairment to a certain degree.

At post-blast day 0, Treated and Untreated rats showed significant threshold shifts in the left and right ears compared to the control group. The Treated group exhibited significant left ear threshold shifts at click and 8-28 kHz, and in the right ear from 8-28 kHz. Untreated rats also exhibited threshold shifts in the left ear at click and 8-28 kHz, and in the right ear from 8-28 kHz ($p < 0.05$).

By 1 week post-blast, significant threshold shifts remained in the left ear at all frequencies and in the right ear at higher frequencies. Treated rats showed threshold shifts in the left ear from 8-28 kHz, and in the right ear at 20 and 28 kHz. The Untreated rats showed threshold shifts in the left ear from 8-28 kHz, and in the right ear at 20 and 28 kHz ($p < 0.05$).

At 3 weeks post-blast, threshold shifts largely recovered in the right ear, but remained at the higher frequencies in the left ear. Treated rats displayed significant threshold shifts at 8 and 16-28 kHz, while Untreated rats showed threshold shifts from 16-28 kHz ($p < 0.05$).

Threshold shifts remained relatively stable from 3 through 6 weeks post-blast. During post-blast week 6, the Treated group exhibited left ear threshold shifts from 8-28 kHz and the Untreated group exhibited shifts from 16-28 kHz ($p < 0.05$).

## Discussion

*Therapeutic effect of sildenafil on tinnitus and hearing loss*

Although sildenafil treatment did not prevent immediate tinnitus onset, it suppressed high-frequency tinnitus from 3 to 6 weeks post-blast, after which high-frequency tinnitus reemerged. Broadband tinnitus followed by high-frequency tinnitus was consistent with our previous report (Mao et al., 2012), except that tinnitus was transient, most likely due to decreased blast exposure parameters (single blast, 14 psi) that were used. Although blast

exposure resulted in both strong startle force reduction and hearing loss during the first week post-blast, startle force remained significantly higher than the noise floor (data not shown) and several studies have reported tinnitus onset following acoustic trauma (Kraus et al., 2010, Norman et al., 2012, Pace and Zhang, 2013), supporting our post-blast week 1 tinnitus findings. As a whole, our current results suggest sildenafil has the capacity to suppress tinnitus, but this occurs in a time-dependent fashion. Several factors may explain why sildenafil was effective at treating high-frequency tinnitus several weeks after blast exposure, but less effective at treating broadband acute tinnitus.

First, immediately following blast, tinnitus-inducing damage to the auditory system may have passed a therapeutic threshold. This is supported by the recovery progression of hearing impairment and the correlation between hearing impairment and tinnitus severity (Goodwin and Johnson, 1980, Hallam et al., 1985). Although sildenafil reduced overall hearing threshold shifts in the right ear at post-blast day 0 and in the left ear at post-blast week 1, it did not significantly reduce left ear threshold shifts at post-blast day 0. The threshold shifts sustained from the latter ear and time point were in the range of 65-80 dB and much higher than the other threshold shifts. This suggests that while sildenafil offers some protection against hearing impairment, it is ineffective past a certain amount of damage. The fact that the left ears of both groups sustained comparable threshold shifts indicates a similar degree of injury. Unilateral noise exposure and unilateral temporary threshold shifts alone are adequate to induce tinnitus and impair gap-detection (Turner et al., 2006, Wang et al., 2009). In the current study, strong tinnitus driven primarily by damage to the left ear may have negated the effect of any reduction in hearing impairment on behavioral performance. In addition, it is possible that sildenafil can only suppress tinnitus once the auditory system has recovered to a certain degree following trauma.

Indeed, sildenafil only began suppressing tinnitus at 3 weeks post-blast, by which time there were no longer any threshold shift differences between Treated and Untreated rats.

Another reason why sildenafil suppressed tinnitus from post-blast week 3-6 but not week 1 may be that acute and more chronic forms of tinnitus have different generators, and these chronic generators are more susceptible to sildenafil treatment. Very little is known about the differences between acute versus chronic generators of tinnitus, in part due to the difficulty of separating acute tinnitus generators from hearing loss and simultaneously confirming tinnitus perception with behavioral testing. Some evidence has suggested that the dorsal cochlear nucleus and inferior colliculus undergo an initial decrease in spontaneous activity immediately after noise exposure, and this hypoactivity transitions to hyperactivity within days to months afterwards (Kaltenbach et al., 2000, Wang et al., 2011). A clinical study that used vardenafil to treat chronic tinnitus, however, found no therapeutic effect (Mazurek et al., 2009), and attributed this to not administering vardenafil sooner after tinnitus-inducing trauma. Therefore, while treating rats with sildenafil immediately after blast exposure did not prevent acute tinnitus, it may have prevented the plastic changes responsible for longer-lasting tinnitus. These plastic changes may have been further modulated by sildenafil treatment during the third week post-blast, but were allowed to take place by post-blast week 7 when high-frequency tinnitus reemerged.

Little research has been conducted on the effect of PDE-5 inhibitors like sildenafil and vardenafil on tinnitus (Mazurek et al., 2009), however, studies have found that they can yield hearing protection and faster hearing recovery from acoustic trauma (Zhang et al., 2011b, Jaumann et al., 2012), as well as adverse effects such as onset hearing impairment (Mukherjee and Shivakumar, 2007, Hong et al., 2008, Maddox et al., 2009, Okuyucu et al., 2009, McGwin, 2010, Snodgrass et al., 2010, Khan et al., 2011, Thakur et al., 2013), though the latter can be

transient (Maddox et al., 2009, Okuyucu et al., 2009). On the contrary, other findings suggest that PDE-5 inhibitors yield no effect on hearing (Mazurek et al., 2009, Giuliano et al., 2010, Thakur et al., 2013). It may be that PDE-5 inhibitors can have negative effects from long-term usage and/or high dosage, but that when administered directly before or after acoustic trauma at a low dosage, they can provide therapeutic effects. PDE-5 inhibitors have been shown to increase Nitric Oxide (NO) and cGMP, which in turn leads to increased vasodilation and blood flow. Intense noise exposure has been shown to reduce partial oxygen pressure and cochlear blood flow (Scheibe et al., 1992, 1993, Lamm and Arnold, 1999), which can at extremes lead to near total degeneration of the inner ear (Ren et al., 1995, Otake et al., 2009). Therefore, initial reduction of hearing impairment and later suppression of high-frequency tinnitus in the Treated group may be related to improved blood flow to the cochlea and peripheral auditory system.

Tinnitus suppression and reduced hearing impairment may have also been due to protective effects from the Akt (Protein Kinase B) pathway and endothelial nitric oxide synthase (eNOS) activation via sildenafil administration. Sildenafil has been shown to activate the Akt pathway, which can enhance neurogenesis following stroke (Wang et al., 2005) and inhibit apoptotic signals, resulting in improved neuronal cell survival and functional recovery following controlled-cortical impact TBI (Noshita et al., 2002, Wu et al., 2011). An immunohistochemistry study has revealed strong staining of phosphorylated-Akt inside and underneath inner hair cells (Hess et al., 2006). At the same time, sildenafil and Akt can increase production of endothelial nitric oxide synthase (eNOS) (Das et al., 2005, Yuan et al., 2008, Shao et al., 2009, Mammi et al., 2011), which has been found in the cochlear microvasculature and spiral ganglia (Gosepath, 1997; Franz, 1996) and has been shown to maintain cerebral blood flow and blood pressure

following controlled cortical impact TBI (Lundblad et al., 2009) and counteract oxidative stress (Chiueh, 1999).

More research is needed to determine if continued sildenafil treatment can result in longer lasting tinnitus suppression. It is currently unclear whether high-frequency tinnitus reemerged due to inefficacy of sildenafil treatment on late-onset tinnitus, or whether tinnitus reemerged due to 3-4 weeks elapsing since the last round of treatment. Additional parameters for exploration include administering sildenafil prior to blast exposure and/or dispensing a stronger dosage to determine if a greater and longer-lasting reduction of tinnitus and hearing impairment can be achieved. Detailed histological work on the peripheral and central auditory system may also be warranted in future studies.

*Therapeutic effect of sildenafil on TBI and hyperacusis-like behavior*

While both Treated and Untreated rats exhibited an initial decrease in startle force during the startle-only condition, the Treated group demonstrated a long-lasting decrease across almost all conditions from the third week onward. In contrast, the Untreated group showed anywhere between little change to occasional decreases and increases in startle force. At the same time, the sham group demonstrated decreased startle force at 1 week post-blast and without background noise at post-blast week 6, although these were isolated instances and most likely reflect natural variability in startle force (i.e. transportation between buildings following pseudo-blast). Initially, these results appear to contradict the therapeutic effects of sildenafil on tinnitus and hearing loss, since they indicate that sildenafil facilitates blast-induced reduction of the startle force. Careful examination of the data, however, may suggest that this is not the case.

Previous studies have shown that both noise exposure and hearing loss (Longenecker and Galazyuk, 2011, Rybalko et al., 2011, Lobarinas et al., 2012), as well as fluid percussion TBI (Wiley et al., 1996, Lu et al., 2003) can reduce acoustic startle force in animals. Although the auditory impact from blast exposure can potentially reduce startle force, this may not be the case in the current study since lasting hearing threshold shifts only occurred at 16-28 kHz in the left ear. Others have indicated that acoustic trauma and hearing loss that primarily affect higher frequency ranges can result in little change to an actual increase in startle force, the latter of which may be due to overrepresentation of lower frequencies and linked to hyperacusis (Ison and Allen, 2003, Ison et al., 2007, Sun et al., 2009, Lu et al., 2011, Sun et al., 2012, Chen et al., 2013a, Pace and Zhang, 2013). Therefore, blast-induced TBI may be a contributing factor to the reduced startle force observed in Treated rats. In humans, blast has been associated with orbitofrontal damage (Mac Donald et al., 2011), which has been linked to reduced startle force (Angrilli et al., 2008). Though we did not measure blast-induced TBI, findings from our previous studies suggesting blast-induced neuroplasticity (Mao et al., 2012) and other studies using similar blasting parameters (1-3 blasts, < 22 psi), including acute mitochondrial dysfunction (Arun et al., 2013), dysregulation in cholinergic and inflammatory pathway-related genes (Valiyaveettil et al., 2013), and disruption of the blood brain barrier (Abdul-Muneer et al., 2013), all suggest the likely induction of TBI in the current study. Furthermore, blasted rats demonstrated a dose-dependent increase in surface righting latency, which has been correlated with TBI severity (Li et al., 2011a, Li et al., 2011b). Although blast injury is known to cause vestibular deficits, including dizziness, disequilibrium, and compromised spatial perception and navigation (Franke et al., 2012), our rats remained completely unconscious prior to regaining

consciousness and righting themselves, suggesting that vestibular defects had limited influence on surface righting latency.

Our results also indicated that sildenafil was ineffective in preventing TBI associated with startle force reduction. Sildenafil has provided physiological and functional treatment against ischemic and embolic stroke (Zhang et al., 2002, Zhang et al., 2005, Zhang et al., 2006) and focal cerebral cryolesion (Li et al., 2007), and can upregulate NO and it's precursor L-arginine, which can enhance functional recovery following percussion TBI (DeWitt et al., 1997, Cherian et al., 1999). It is possible then that sildenafil may be effective on hypoxia- and ischemia-induced TBI, but not on TBI associated with reduced startle force. To our knowledge, this is the first study to explore the interactions between blast exposure, sildenafil, and startle force. More detailed work is needed to determine whether sildenafil has a neuroprotective effect on blast-induced hypoxic and ischemic injury, and whether certain injury can be associated with startle reflex performance.

While complicated, the little change to occasional decreased and increased startle force in the Untreated group from post-blast week 3 onward may suggest the presence of both TBI and hyperacusis-like behavior. Since sildenafil suppressed tinnitus in the Treated group, it is possible that sildenafil also suppressed hyperacusis development, given the high correlation (Turner and Parrish, 2008, Turner et al., 2012, Chen et al., 2013a, Pace and Zhang, 2013) and putatively similarly shared pathophysiology between tinnitus and hyperacusis (Nelson and Chen, 2004, Attias et al., 2005, Moller, 2007, Sun et al., 2012). Others have shown that noise exposure can result in increased startle responsivity (Sun et al., 2012, Chen et al., 2013a, Pace and Zhang, 2013), and improved PPI (Turner and Parrish, 2008, Turner et al., 2012), which are attributed to hyperacusis-like behavior. The latter may be related to the Untreated group during post-blast

week 6, where 10-12 and 14-16 kHz PPI ratios were transiently better than the Treated group. Thus, while TBI may have served to reduce startle force, the simultaneous presence of hyperacusis-like percepts may have increased startle responsivity in the Untreated group, resulting in the little change to occasional increases and decreases seen in their startle amplitude. To our knowledge, this is the first animal study that shows a potential relationship between blast exposure and hyperacusis. Additional studies with a longer observational period are needed to better understand and establish this relationship.

## Conclusions

Sildenafil treatment reduced behavioral evidence of high-frequency tinnitus, as well as a certain degree of hearing impairment and hyperacusis-like behavior. These therapeutic effects are likely due to vasodilation and improved blood flow to the peripheral and central auditory system, which disrupted the normative and potentially maladaptive plastic changes following acoustic trauma. In spite of these effects, sildenafil treatment appeared to be limited to a certain degree of tinnitus- and hearing-related damage and did not prevent startle force reduction, which may have been caused by blast-induced TBI. Taken together, our results suggest that sildenafil can help attenuate adverse auditory consequences of blast exposure. More research is needed, however, to determine if sildenafil can yield greater and longer-lasting reduction of tinnitus and hearing impairment, and even their associated TBI. Increasing sildenafil dosage, as well as administering sildenafil prior to blast exposure, may be promising treatment routes, in addition to combination with other approaches such as electrical stimulation and sound therapy to optimally modulate the underlying pathological neuroplasticity. Given the considerable physical and

psychological detriments associated with blast-induced tinnitus, auditory impairment, and TBI, there is an urgent need for clearer understanding of the overall problem and improved treatment outcomes.

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## Figure Legends

**Figure 1.** Photograph showing the Wayne State University shock tube apparatus with close-up of pressure transducer and rat holder.

**Figure 2.** Surface to right latency following each of the three blast or pseudo-blast exposures in the Treated, Untreated and Sham groups. Although the Treated group showed a significant latency increase after the second blast compared to the first, both the Treated and Untreated groups demonstrated similarly increased surface righting latencies after the third blast-exposure. Notably, the Sham group showed no difference in surface righting latency after any of the three pseudo-blast exposures.

**Figure 3.** Data showing percent change (from baseline) of GAP and PPI ratios for the Treated, Untreated, and Sham groups during post-blast week 1, 3, 4, 6, and 7 **(A-J)**. During post-blast week 1, both the Treated and Untreated groups showed significant upward percent change across all GAP ratio frequencies, indicating tinnitus **(A)**. Both groups

also showed significant upward change across all PPI ratio frequencies, indicating auditory detection deficits, however the Untreated group exhibited stronger deficits at several frequencies **(B)**. By post-blast week 3, the Untreated group demonstrated tinnitus presence at 18-20 kHz and particularly robust tinnitus at 26-28 kHz **(C)**, while the Treated group tinnitus showed suppression at 26-28 kHz, despite tinnitus presence at 6-8 and 18-20 kHz. In contrast, although both groups displayed auditory detection deficits from 10-18 kHz **(D)**, the Treated group showed a greater deficit at 14-16 kHz. At post-blast week 4, the Untreated group showed tinnitus presence from 14-28 kHz and BBN, while the Treated group only showed tinnitus at 18-20 kHz **(E)**. Auditory detection deficits were seen from 8-20 kHz in the Untreated group at post-blast week 4, and from 10-28 kHz and BBN in the Treated group **(F)**. At 6 weeks post-blast, the Untreated group exhibited tinnitus at 14-16 and 26-28 kHz, while the Treated group showed tinnitus at 14-16 and 18-20 kHz and suppression at 26-28 kHz **(G)**. The Untreated group, however, showed auditory detection deficits at 18-20 kHz **(H)**. The Treated group showed auditory detection deficits from 10-20 kHz. By the 7 week post-blast time point, the Untreated group retained 26-28 kHz tinnitus while 6-8 and 26-28 kHz tinnitus reemerged in the Treated group **(I)**. Both the Untreated and Treated groups displayed auditory detection deficits from 6-20 kHz, with the Treated group also showing deficits at 26-28 kHz and BBN **(J)**.

**Figure 4.** Percent change (from baseline) of startle force in response to the startle only condition with background noise (gap-detection) and without PPI for the Treated, Untreated, and Sham groups during post-blast week 1, 3, 4, 6, and 7 **(A-J)**. During the first week post-blast, both Untreated and Treated groups showed significant startle force decrease in

response to the startle only condition with **(A)** and without **(B)** background noise. By post-blast week 3, the Untreated group only showed a startle force decrease during 14-16 kHz background noise while the Treated group showed decrease during all frequencies **(C)**. In the absence of background noise, the Untreated group showed little change except for an increase near 6-8 kHz and BBN prepulses, whereas the Treated group showed decreases near all frequencies **(D)**. At post-blast week 4, the Untreated group demonstrated no startle force decreases during background noise **(E)** and increased startle force near 26-28 kHz and BBN prepulses **(F)**, while the Treated group demonstrated decreases across all frequencies during and without background noise **(E-F)**. At 6 weeks post-blast, the Untreated group only showed decreased startle force during 14-16 kHz background noise **(G)**, but showed significantly greater startle force than the Sham or Treated groups in the absence of background noise **(H)**. The Treated group showed decreased startle force during all frequencies of background noise **(G)** but similar startle force to the Sham group in the absence of background noise **(H)**. Seven-week post-blast data reveal decreased startle force in the Untreated group from 10-28 kHz and in the Treated group during all background noise frequencies **(I)**, as well as an increase in startle force near 26-28 kHz prepulses for the Untreated group and decreased startle force across all conditions for the Treated group **(J)**.

**Figure 5.** ABR threshold shifts obtained during post-blast day 0, post-blast weeks 1, 3, and 6 for the Treated, Untreated, and Sham groups in the exposed left ear **(A)** and plugged right ear **(B)**. At post-blast day 0, significant threshold shifts averaging between 55-80 dB were observed in the exposed ear of the Treated and Untreated groups in clicks and

tone bursts **(A)**. The occluded ears with ear plugs still sustained significant threshold shifts across all tone burst frequencies, however, the Treated group demonstrated an overall decrease in frequency threshold shifts compared to the Untreated group **(B)**. By post-blast week 1, the Treated group exhibited an overall decrease in frequency threshold shifts compared to the Untreated group in the exposed ear **(A)**. While both groups still exhibited significant threshold shifts in the plugged ear **(B)**, there were no longer significant differences between groups. From the third week post-blast onward, threshold shifts recovered for both groups in the occluded ear **(B)**. In the exposed ear, significant threshold shifts were observed from 16-28 kHz during post-blast week 3 and 6 for the Untreated group, and in the Treated group at 8 and 16-28 kHz during post-blast week 3 and at all frequencies during post-blast week 6 **(A)**. There were, however, no significant differences between the Treated and Untreated groups at post-blast weeks 3 and 6.

## References Cited:

Abdul-Muneer PM, Schuetz H, Wang F, Skotak M, Jones J, Gorantla S, Zimmerman MC, Chandra N, Haorah J (2013) Induction of oxidative and nitrosative damage leads to cerebrovascular inflammation in an animal model of mild traumatic brain injury induced by primary blast. Free radical biology & medicine 60:282-291.

Administration VB (2011) Annual Benefits Report. US Department of Veterans Affairs.

Angrilli A, Bianchin M, Radaelli S, Bertagnoni G, Pertile M (2008) Reduced startle reflex and aversive noise perception in patients with orbitofrontal cortex lesions. Neuropsychologia 46:1179-1184.

Arun P, Abu-Taleb R, Oguntayo S, Wang Y, Valiyaveettil M, Long JB, Nambiar MP (2013) Acute Mitochondrial Dysfunction after Blast Exposure: Potential Role of Mitochondrial Glutamate Oxaloacetate Transaminase. J Neurotrauma.

Attias J, Zwecker-Lazar I, Nageris B, Keren O, Groswasser Z (2005) Dysfunction of the auditory efferent system in patients with traumatic brain injuries with tinnitus and hyperacusis. JBasic ClinPhysiol Pharmacol 16:117-126.

Baguley DM, Jones S, Wilkins I, Axon PR, Moffat DA (2005) The inhibitory effect of intravenous lidocaine infusion on tinnitus after translabyrinthine removal of vestibular schwannoma: a double-blind, placebo-controlled, crossover study. OtolNeurotol 26:169-176.

Baldo P, Doree C, Lazzarini R, Molin P, McFerran DJ (2006) Antidepressants for patients with tinnitus. Cochrane Database Syst Rev CD003853.

Bauer CA, Brozoski TJ (2001) Assessing tinnitus and prospective tinnitus therapeutics using a psychophysical animal model. JAssocResOtolaryngol 2:54-64.

Bauer CA, Brozoski TJ (2006) Effect of gabapentin on the sensation and impact of tinnitus. Laryngoscope 116:675-681.

Bauer CA, Turner JG, Caspary DM, Myers KS, Brozoski TJ (2008) Tinnitus and inferior colliculus activity in chinchillas related to three distinct patterns of cochlear trauma. J NeurosciRes.

Cahani M, Paul G, Shahar A (1983) Tinnitus pitch and acoustic trauma. Audiology 22:357-363.

Cazals Y (2000) Auditory sensori-neural alterations induced by salicylate. Prog Neurobiol 62:583-631.

Chen G, Lee C, Sandridge SA, Butler HM, Manzoor NF, Kaltenbach JA (2013a) Behavioral evidence for possible simultaneous induction of hyperacusis and tinnitus following intense sound exposure. J Assoc Res Otolaryngol 14:413-424.

Chen GD, Jastreboff PJ (1995) Salicylate-induced abnormal activity in the inferior colliculus of rats. HearRes 82:158-178.

Chen W, Wang J, Chen J, Chen J, Chen Z (2013b) Relationship between changes in the cochlear blood flow and disorder of hearing function induced by blast injury in guinea pigs. International journal of clinical and experimental pathology 6:375-384.

Cherian L, Chacko G, Goodman JC, Robertson CS (1999) Cerebral hemodynamic effects of phenylephrine and L-arginine after cortical impact injury. Crit Care Med 27:2512-2517.

Crocetti A, Forti S, Ambrosetti U, Bo LD (2009) Questionnaires to evaluate anxiety and depressive levels in tinnitus patients. OtolaryngolHead Neck Surg 140:403-405.

Das A, Xi L, Kukreja RC (2005) Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. J Biol Chem 280:12944-12955.

DeWitt DS, Smith TG, Deyo DJ, Miller KR, Uchida T, Prough DS (1997) L-arginine and superoxide dismutase prevent or reverse cerebral hypoperfusion after fluid-percussion traumatic brain injury. J Neurotrauma 14:223-233.

Eggermont JJ (2007) Correlated neural activity as the driving force for functional changes in auditory cortex. HearRes 229:69-80.

Eggermont JJ, Roberts LE (2004) The neuroscience of tinnitus. Trends Neurosci 27:676-682.

Ewert DL, Lu J, Li W, Du X, Floyd R, Kopke R (2012) Antioxidant treatment reduces blast-induced cochlear damage and hearing loss. Hear Res 285:29-39.

Finlayson PG, Kaltenbach JA (2009) Alterations in the spontaneous discharge patterns of single units in the dorsal cochlear nucleus following intense sound exposure. HearRes 256:104-117.

Franke LM, Walker WC, Cifu DX, Ochs AL, Lew HL (2012) Sensorintegrative dysfunction underlying vestibular disorders after traumatic brain injury: a review. J Rehabil Res Dev 49:985-994.

Giuliano F, Jackson G, Montorsi F, Martin-Morales A, Raillard P (2010) Safety of sildenafil citrate: review of 67 double-blind placebo-controlled trials and the postmarketing safety database. International journal of clinical practice 64:240-255.

Gondusky JS, Reiter MP (2005) Protecting military convoys in Iraq: an examination of battle injuries sustained by a mechanized battalion during Operation Iraqi Freedom II. Mil Med 170:546-549.

Goodwin PE, Johnson RM (1980) The loudness of tinnitus. Acta Otolaryngol 90:353-359.

Hallam RS, Jakes SC, Chambers C, Hinchcliffe R (1985) A comparison of different methods for assessing the 'intensity' of tinnitus. Acta Otolaryngol 99:501-508.

Hasson D, Theorell T, Benka WM, Leineweber C, Canlon B (2011) Stress and prevalence of hearing problems in the Swedish working population. BMCPublic Health 11:130.

Hebert S, Fullum S, Carrier J (2011) Polysomnographic and quantitative electroencephalographic correlates of subjective sleep complaints in chronic tinnitus. JSleep Res 20:38-44.

Hess A, Labbe D, Watanabe K, Bloch W, Michel O (2006) Evidence for an Akt-kinase/NO/cGMP pathway in the cochlea of guinea pigs. Eur Arch Otorhinolaryngol 263:75-78.

Hesser H, Andersson G (2009) The role of anxiety sensitivity and behavioral avoidance in tinnitus disability. IntJAudiol 48:295-299.

Hiller W, Goebel G (1999) Assessing audiological, pathophysiological, and psychological variables in chronic tinnitus: a study of reliability and search for prognostic factors. IntJBehavMed 6:312-330.

Hong BN, Yi TH, Kim SY, Kang TH (2008) High dosage sildenafil induces hearing impairment in mice. Biological & pharmaceutical bulletin 31:1981-1984.

Hue CD, Cao S, Haider SF, Vo KV, Effgen GB, Vogel E, 3rd, Panzer MB, Bass CR, Meaney DF, Morrison B, 3rd (2013) Blood-brain barrier dysfunction after primary blast injury in vitro. J Neurotrauma 30:1652-1663.

Ison JR, Allen PD (2003) Low-frequency tone pips elicit exaggerated startle reflexes in C57BL/6J mice with hearing loss. Journal of the Association for Research in Otolaryngology : JARO 4:495-504.

Ison JR, Allen PD, O'Neill WE (2007) Age-related hearing loss in C57BL/6J mice has both frequency-specific and non-frequency-specific components that produce a hyperacusis-like exaggeration of the acoustic startle reflex. JAssocResOtolaryngol 8:539-550.

Jastreboff PJ, Brennan JF, Coleman JK, Sasaki CT (1988) Phantom auditory sensation in rats: an animal model for tinnitus. BehavNeurosci 102:811-822.

Jastreboff PJ, Brennan JF, Sasaki CT (1991) Quinine-induced tinnitus in rats. ArchOtolaryngolHead Neck Surg 117:1162-1166.

Jaumann M, Dettling J, Gubelt M, Zimmermann U, Gerling A, Paquet-Durand F, Feil S, Wolpert S, Franz C, Varakina K, Xiong H, Brandt N, Kuhn S, Geisler HS, Rohbock K, Ruth P, Schlossmann J, Hutter J, Sandner P, Feil R, Engel J, Knipper M, Ruttiger L (2012) cGMP-Prkg1 signaling and Pde5 inhibition shelter cochlear hair cells and hearing function. Nat Med 18:252-259.

Kaltenbach JA, Rachel JD, Mathog TA, Zhang J, Falzarano PR, Lewandowski M (2002) Cisplatin-induced hyperactivity in the dorsal cochlear nucleus and its relation to outer hair cell loss: relevance to tinnitus. JNeurophysiol 88:699-714.

Kaltenbach JA, Zhang J, Afman CE (2000) Plasticity of spontaneous neural activity in the dorsal cochlear nucleus after intense sound exposure. HearRes 147:282-292.

Khan AS, Sheikh Z, Khan S, Dwivedi R, Benjamin E (2011) Viagra deafness--sensorineural hearing loss and phosphodiesterase-5 inhibitors. Laryngoscope 121:1049-1054.

Kimura M, Eggermont JJ (1999) Effects of acute pure tone induced hearing loss on response properties in three auditory cortical fields in cat. HearRes 135:146-162.

Kraus KS, Ding D, Jiang H, Lobarinas E, Sun W, Salvi RJ (2011) Relationship between noise-induced hearing-loss, persistent tinnitus and growth-associated protein-43 expression in the rat cochlear nucleus: does synaptic plasticity in ventral cochlear nucleus suppress tinnitus? Neuroscience 194:309-325.

Kraus KS, Mitra S, Jimenez Z, Hinduja S, Ding D, Jiang H, Gray L, Lobarinas E, Sun W, Salvi RJ (2010) Noise trauma impairs neurogenesis in the rat hippocampus. Neuroscience 167:1216-1226.

Kujawa SG, Liberman MC (2009) Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. J Neurosci 29:14077-14085.

Lamm K, Arnold W (1999) Successful treatment of noise-induced cochlear ischemia, hypoxia, and hearing loss. Ann N Y Acad Sci 884:233-248.

Langguth B, Elgoyhen AB (2012) Current pharmacological treatments for tinnitus. Expert opinion on pharmacotherapy 13:2495-2509.

Lenarz T (1986) Treatment of tinnitus with lidocaine and tocainide. ScandAudiolSuppl 26:49-51.

Lew HL, Jerger JF, Guillory SB, Henry JA (2007) Auditory dysfunction in traumatic brain injury. J Rehabil Res Dev 44:921-928.

Li L, Jiang Q, Zhang L, Ding G, Gang Zhang Z, Li Q, Ewing JR, Lu M, Panda S, Ledbetter KA, Whitton PA, Chopp M (2007) Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. Brain Res 1132:185-192.

Li Y, Zhang L, Kallakuri S, Zhou B, Cavanaugh JM (2011a) Injury Predictors for Traumatic Axonal Injury in a Rodent Head Impact Acceleration Model. Stapp Car Crash J 55:25-47.

Li Y, Zhang L, Kallakuri S, Zhou R, Cavanaugh JM (2011b) Quantitative relationship between axonal injury and mechanical response in a rodent head impact acceleration model. Journal of Neurotrauma 28:1767-1782.

Lobarinas E, Hayes SH, Allman BL (2012) The gap-startle paradigm for tinnitus screening in animal models: Limitations and optimization. Hear Res.

Lobarinas E, Yang G, Sun W, Ding D, Mirza N, Dalby-Brown W, Hilczmayer E, Fitzgerald S, Zhang L, Salvi R (2006) Salicylate- and quinine-induced tinnitus and effects of memantine. Acta OtolaryngolSuppl 13-19.

Long JB, Bentley TL, Wessner KA, Cerone C, Sweeney S, Bauman RA (2009) Blast overpressure in rats: recreating a battlefield injury in the laboratory. J Neurotrauma 26:827-840.

Longenecker RJ, Galazyuk AV (2011) Development of tinnitus in CBA/CaJ mice following sound exposure. J Assoc Res Otolaryngol 12:647-658.

Lu J, Lobarinas E, Deng A, Goodey R, Stolzberg D, Salvi RJ, Sun W (2011) GABAergic neural activity involved in salicylate-induced auditory cortex gain enhancement. Neuroscience 189:187-198.

Lu J, Moochhala S, Shirhan M, Ng KC, Teo AL, Tan MH, Moore XL, Wong MC, Ling EA (2003) Neuroprotection by aminoguanidine after lateral fluid-percussive brain injury in rats: a combined magnetic resonance imaging, histopathologic and functional study. Neuropharmacology 44:253-263.

Lundblad C, Grande PO, Bentzer P (2009) Hemodynamic and histological effects of traumatic brain injury in eNOS-deficient mice. J Neurotrauma 26:1953-1962.

Luo H, Zhang X, Nation J, Pace E, Lepczyk L, Zhang J (2012) Tinnitus suppression by electrical stimulation of the rat dorsal cochlear nucleus. Neurosci Lett.

Ma W-LD, Hidaka H, May BJ (2006) Spontaneous activity in the inferior colliculus of CBA/J mice after manipulations that induce tinnitus. Hearing Research 212:9-21.

Mac Donald CL, Johnson AM, Cooper D, Nelson EC, Werner NJ, Shimony JS, Snyder AZ, Raichle ME, Witherow JR, Fang R, Flaherty SF, Brody DL (2011) Detection of blast-related traumatic brain injury in U.S. military personnel. N Engl J Med 364:2091-2100.

Maddox PT, Saunders J, Chandrasekhar SS (2009) Sudden hearing loss from PDE-5 inhibitors: A possible cellular stress etiology. Laryngoscope 119:1586-1589.

Mahlke C, Wallhausser-Franke E (2004) Evidence for tinnitus-related plasticity in the auditory and limbic system, demonstrated by arg3.1 and c-fos immunocytochemistry. HearRes 195:17-34.

Mammi C, Pastore D, Lombardo MF, Ferrelli F, Caprio M, Consoli C, Tesauro M, Gatta L, Fini M, Federici M, Sbraccia P, Donadel G, Bellia A, Rosano GM, Fabbri A, Lauro D (2011) Sildenafil reduces insulin-resistance in human endothelial cells. PLoS One 6:e14542.

Man A, Naggan L (1981) Characteristics of tinnitus in acoustic trauma. Audiology 20:72-78.

Mao JC, Pace E, Pierozynski P, Kou Z, Shen Y, Vandevord P, Haacke EM, Zhang X, Zhang J (2012) Blast-induced tinnitus and hearing loss in rats: behavioral and imaging assays. J Neurotrauma 29:430-444.

Mazurek B, Haupt H, Szczepek AJ, Sandmann J, Gross J, Klapp BF, Kiesewetter H, Kalus U, Stover T, Caffier PP (2009) Evaluation of vardenafil for the treatment of subjective tinnitus: a controlled pilot study. J Negat Results Biomed 8:3.

McGwin G, Jr. (2010) Phosphodiesterase type 5 inhibitor use and hearing impairment. Arch Otolaryngol Head Neck Surg 136:488-492.

Moller AR (2007) Tinnitus: presence and future. ProgBrain Res 166:3-16.

Mukherjee B, Shivakumar T (2007) A case of sensorineural deafness following ingestion of sildenafil. J Laryngol Otol 121:395-397.

Myers SA, Devries WH, Gruenthal MJ, Andres KR, Hagg T, Whittemore SR (2011) Sildenafil Improves Epicenter Vascular Perfusion but not Hindlimb Functional Recovery after Contusive Spinal Cord Injury in Mice. J Neurotrauma.

Nelson JJ, Chen K (2004) The relationship of tinnitus, hyperacusis, and hearing loss. Ear Nose Throat J 83:472-476.

Norman M, Tomscha K, Wehr M (2012) Isoflurane blocks temporary tinnitus. Hear Res 290:64-71.

Noshita N, Lewen A, Sugawara T, Chan PH (2002) Akt phosphorylation and neuronal survival after traumatic brain injury in mice. Neurobiol Dis 9:294-304.

Nowotny M, Remus M, Kossl M, Gaese BH (2011) Characterization of the perceived sound of trauma-induced tinnitus in gerbils. J Acoust Soc Am 130:2827-2834.

Oishi N, Kanzaki S, Shinden S, Saito H, Inoue Y, Ogawa K (2010) Effects of selective serotonin reuptake inhibitor on treating tinnitus in patients stratified for presence of depression or anxiety. AudiolNeurootol 15:187-193.

Okuyucu S, Guven OE, Akoglu E, Ucar E, Dagli S (2009) Effect of phosphodiesterase-5 inhibitor on hearing. J Laryngol Otol 123:718-722.

Otake H, Yamamoto H, Teranishi M, Sone M, Nakashima T (2009) Cochlear blood flow during occlusion and reperfusion of the anterior inferior cerebellar artery--effect of topical application of dexamethasone to the round window. Acta Otolaryngol 129:127-131.

Pace E, Zhang J.S. (2013) Noise-Induced Tinnitus Using Individualized Gap Detection Analysis and Its Relationship with Hyperacusis, Anxiety, and Spatial Cognition. PLoS ONE 8:e75011.

Peiffer AM, Friedman JT, Rosen GD, Fitch RH (2004) Impaired gap detection in juvenile microgyric rats. Brain Res Dev Brain Res 152:93-98.

Prabhu LV, Rai R, Ranade AV, Kini H, Krishnamurthy A, Bernhardt KL (2013) Effect of sildenafil-induced nitric oxide on the histomorphology of cardiomyocytes in male rats. International journal of applied & basic medical research 3:84-87.

Ren T, Brown NJ, Zhang M, Nuttall AL, Miller JM (1995) A reversible ischemia model in gerbil cochlea. Hear Res 92:30-37.

Reyes SA, Salvi RJ, Burkard RF, Coad ML, Wack DS, Galantowicz PJ, Lockwood AH (2002) Brain imaging of the effects of lidocaine on tinnitus. HearRes 171:43-50.

Rossiter S, Stevens C, Walker G (2006) Tinnitus and its effect on working memory and attention. JSpeech Lang HearRes 49:150-160.

Rybalko N, Bures Z, Burianova J, Popelar J, Grecova J, Syka J (2011) Noise exposure during early development influences the acoustic startle reflex in adult rats. Physiology & behavior 102:453-458.

Sajja VS, Galloway MP, Ghoddoussi F, Thiruthalinathan D, Kepsel A, Hay K, Bir CA, Vandevord PJ (2012) Blast-induced neurotrauma leads to neurochemical changes and neuronal degeneration in the rat hippocampus. NMR Biomed 25:1331-1339.

Scheibe F, Haupt H, Ludwig C (1992) Intensity-dependent changes in oxygenation of cochlear perilymph during acoustic exposure. Hear Res 63:19-25.

Scheibe F, Haupt H, Ludwig C (1993) Intensity-related changes in cochlear blood flow in the guinea pig during and following acoustic exposure. Eur Arch Otorhinolaryngol 250:281-285.

Seki S, Eggermont JJ (2003) Changes in spontaneous firing rate and neural synchrony in cat primary auditory cortex after localized tone-induced hearing loss. HearRes 180:28-38.

Shao ZH, Wojcik KR, Dossumbekova A, Hsu C, Mehendale SR, Li CQ, Qin Y, Sharp WW, Chang WT, Hamann KJ, Yuan CS, Hoek TL (2009) Grape seed proanthocyanidins protect cardiomyocytes from ischemia and reperfusion injury via Akt-NOS signaling. J Cell Biochem 107:697-705.

Shargorodsky J, Curhan GC, Farwell WR (2010) Prevalence and characteristics of tinnitus among US adults. The American journal of medicine 123:711-718.

Shin HS, Bae SK, Lee MG (2006) Pharmacokinetics of sildenafil after intravenous and oral administration in rats: hepatic and intestinal first-pass effects. Int J Pharm 320:64-70.

Shulman A, Strashun AM, Afriyie M, Aronson F, Abel W, Goldstein B (1995) SPECT Imaging of Brain and Tinnitus-Neurotologic/Neurologic Implications. IntTinnitusJ 1:13-29.

Snodgrass AJ, Campbell HM, Mace DL, Faria VL, Swanson KM, Holodniy M (2010) Sudden sensorineural hearing loss associated with vardenafil. Pharmacotherapy 30:112.

Solt K, Cotten JF, Cimenser A, Wong KF, Chemali JJ, Brown EN (2011) Methylphenidate actively induces emergence from general anesthesia. Anesthesiology 115:791-803.

Stevens C, Walker G, Boyer M, Gallagher M (2007) Severe tinnitus and its effect on selective and divided attention. IntJAudiol 46:208-216.

Sun W, Deng A, Jayaram A, Gibson B (2012) Noise exposure enhances auditory cortex responses related to hyperacusis behavior. Brain Res 1485:108-116.

Sun W, Lu J, Stolzberg D, Gray L, Deng A, Lobarinas E, Salvi RJ (2009) Salicylate increases the gain of the central auditory system. Neuroscience 159:325-334.

Svetlov SI, Prima V, Kirk DR, Gutierrez H, Curley KC, Hayes RL, Wang KK (2010) Morphologic and biochemical characterization of brain injury in a model of controlled blast overpressure exposure. The Journal of trauma 69:795-804.

Swetter BJ, Fitch RH, Markus EJ (2010) Age-related decline in auditory plasticity: experience dependent changes in gap detection as measured by prepulse inhibition in young and aged rats. Behav Neurosci 124:370-380.

Temmel AF, Kierner AC, Steurer M, Riedl S, Innitzer J (1999) Hearing loss and tinnitus in acute acoustic trauma. WienKlinWochenschr 111:891-893.

Test T, Canfi A, Eyal A, Shoam-Vardi I, Sheiner EK (2011) The influence of hearing impairment on sleep quality among workers exposed to harmful noise. Sleep 34:25-30.

Thakur JS, Thakur S, Sharma DR, Mohindroo NK, Thakur A, Negi PC (2013) Hearing loss with phosphodiesterase-5 inhibitors: a prospective and objective analysis with tadalafil. Laryngoscope 123:1527-1530.

Turner J, Larsen D, Hughes L, Moechars D, Shore S (2012) Time course of tinnitus development following noise exposure in mice. J Neurosci Res.

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, Hughes LF, Caspary DM (2006) Gap detection deficits in rats with tinnitus: a potential novel screening tool. BehavNeurosci 120:188-195.

Turner JG, Parrish J (2008) Gap detection methods for assessing salicylate-induced tinnitus and hyperacusis in rats. Am J Audiol 17:S185-192.

Valiyaveettil M, Alamneh Y, Miller SA, Hammamieh R, Wang Y, Arun P, Wei Y, Oguntayo S, Nambiar MP (2012) Preliminary studies on differential expression of auditory functional genes in the brain after repeated blast exposures. J Rehabil Res Dev 49:1153-1162.

Valiyaveettil M, Alamneh YA, Miller SA, Hammamieh R, Arun P, Wang Y, Wei Y, Oguntayo S, Long JB, Nambiar MP (2013) Modulation of cholinergic pathways and inflammatory mediators in blast-induced traumatic brain injury. Chemico-biological interactions 203:371-375.

Vogler DP, Robertson D, Mulders WH (2011) Hyperactivity in the ventral cochlear nucleus after cochlear trauma. J Neurosci 31:6639-6645.

Wallhausser-Franke E, Mahlke C, Oliva R, Braun S, Wenz G, Langner G (2003) Expression of c-fos in auditory and non-auditory brain regions of the gerbil after manipulations that induce tinnitus. ExpBrain Res 153:649-654.

Wang H, Brozoski TJ, Caspary DM (2011) Inhibitory neurotransmission in animal models of tinnitus: maladaptive plasticity. Hear Res 279:111-117.

Wang H, Brozoski TJ, Turner JG, Ling L, Parrish JL, Hughes LF, Caspary DM (2009) Plasticity at glycinergic synapses in dorsal cochlear nucleus of rats with behavioral evidence of tinnitus. Neuroscience 164:747-759.

Wang H, Tian J, Yin D (2000) [Positron emission tomography of tinnitus-related brain areas]. Zhonghua ErBi YanHou KeZa Zhi 35:420-424.

Wang L, Gang Zhang Z, Lan Zhang R, Chopp M (2005) Activation of the PI3-K/Akt pathway mediates cGMP enhanced-neurogenesis in the adult progenitor cells derived from the subventricular zone. J Cereb Blood Flow Metab 25:1150-1158.

Wiley JL, Compton AD, Pike BR, Temple MD, McElderry JW, Hamm RJ (1996) Reduced sensorimotor reactivity following traumatic brain injury in rats. Brain Res 716:47-52.

Wu Y, Xia ZY, Meng QT, Zhu J, Lei S, Xu J, Dou J (2011) Shen-Fu injection preconditioning inhibits myocardial ischemia-reperfusion injury in diabetic rats: activation of eNOS via the PI3K/Akt pathway. J Biomed Biotechnol 2011:384627.

Yang G, Lobarinas E, Zhang L, Turner J, Stolzberg D, Salvi R, Sun W (2007) Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. HearRes 226:244-253.

Yuan Z, Hein TW, Rosa RH, Jr., Kuo L (2008) Sildenafil (Viagra) evokes retinal arteriolar dilation: dual pathways via NOS activation and phosphodiesterase inhibition. Invest Ophthalmol Vis Sci 49:720-725.

Zhang J, Zhang Y, Zhang X (2011a) Auditory cortex electrical stimulation suppresses tinnitus in rats. J Assoc Res Otolaryngol 12:185-201.

Zhang JS, Kaltenbach JA (1998) Increases in spontaneous activity in the dorsal cochlear nucleus of the rat following exposure to high-intensity sound. Neurosci Lett 250:197-200.

Zhang JS, Kaltenbach JA, Wang J, Kim SA (2003) Fos-like immunoreactivity in auditory and nonauditory brain structures of hamsters previously exposed to intense sound. ExpBrain Res 153:655-660.

Zhang L, Zhang RL, Wang Y, Zhang C, Zhang ZG, Meng H, Chopp M (2005) Functional recovery in aged and young rats after embolic stroke: treatment with a phosphodiesterase type 5 inhibitor. Stroke 36:847-852.

Zhang L, Zhang Z, Zhang RL, Cui Y, LaPointe MC, Silver B, Chopp M (2006) Tadalafil, a long-acting type 5 phosphodiesterase isoenzyme inhibitor, improves neurological functional recovery in a rat model of embolic stroke. Brain Res 1118:192-198.

Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M, Chopp M (2002) Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. Stroke 33:2675-2680.

Zhang SJ, Zhang X, Liang Y, Yue ZL (2011b) [Effect of sildenafil on ABR thresholds shift to noise-induced hearing loss in guinea pigs]. Zhonghua er bi yan hou tou jing wai ke za zhi = Chinese journal of otorhinolaryngology head and neck surgery 46:844-847.

Zheng Y, Vagal S, McNamara E, Darlington CL, Smith PF (2012) A dose-response analysis of the effects of L-baclofen on chronic tinnitus caused by acoustic trauma in rats. Neuropharmacology 62:940-946.

Figure 1
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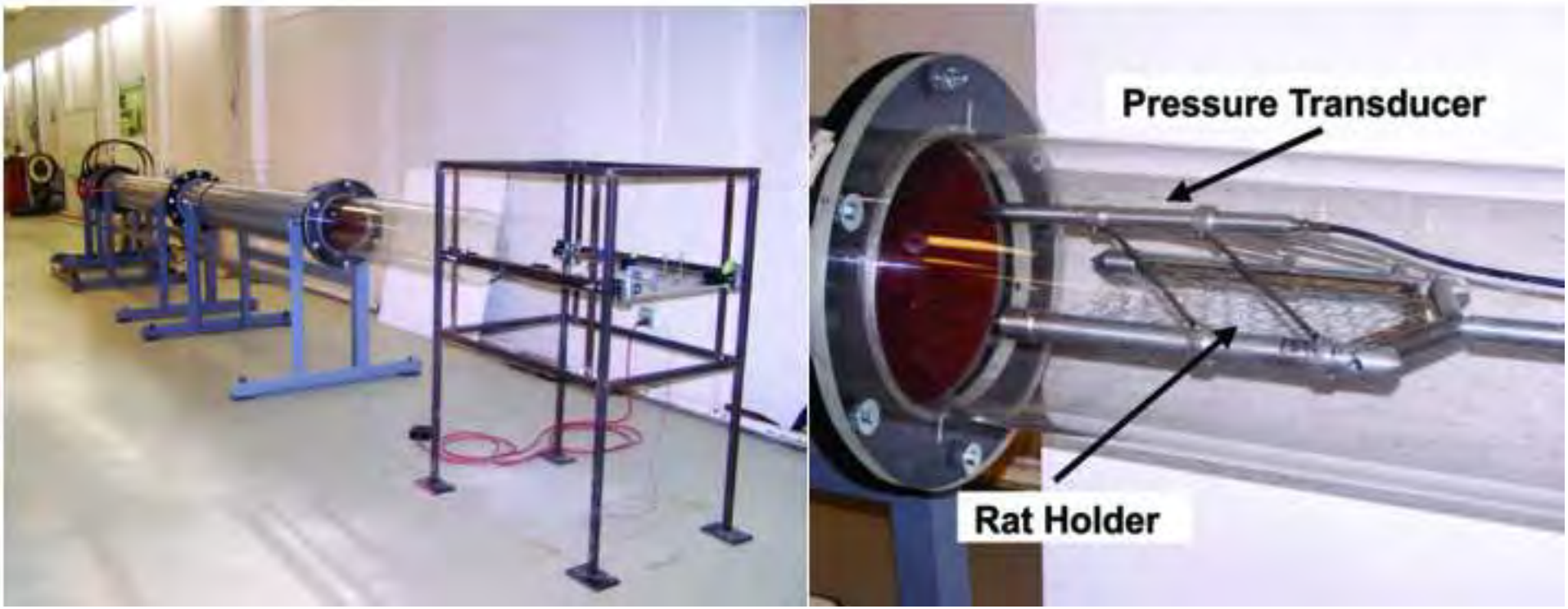
# Figure 1



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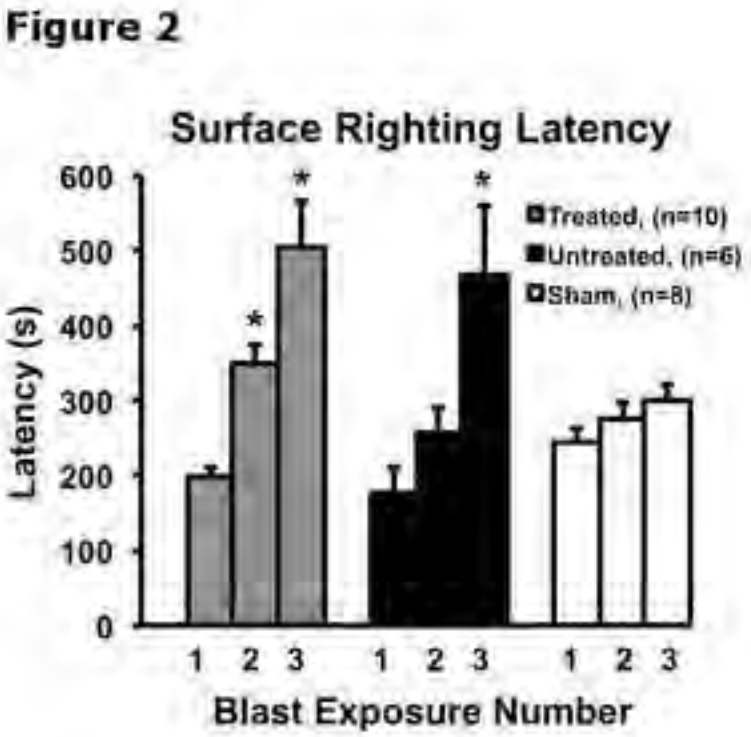

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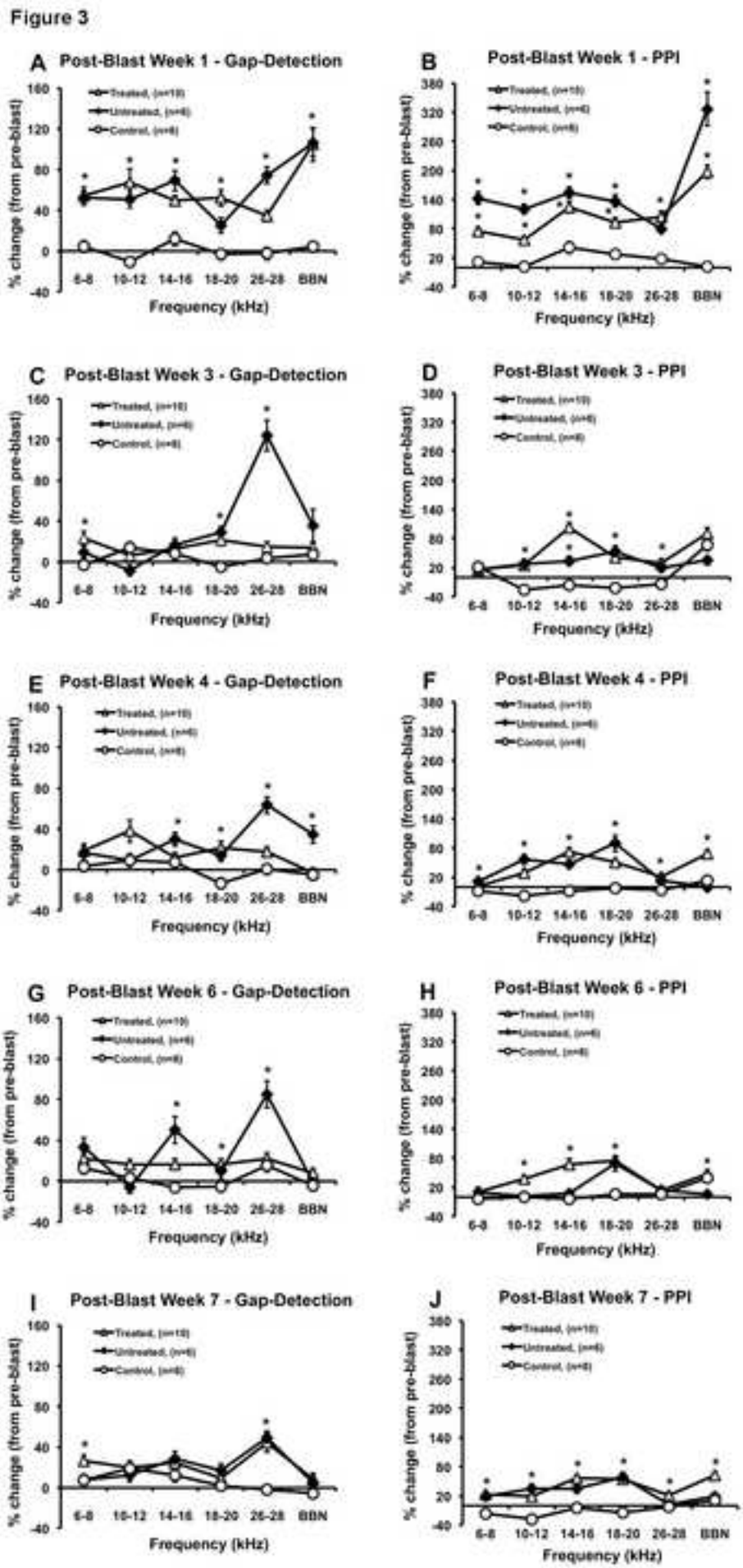

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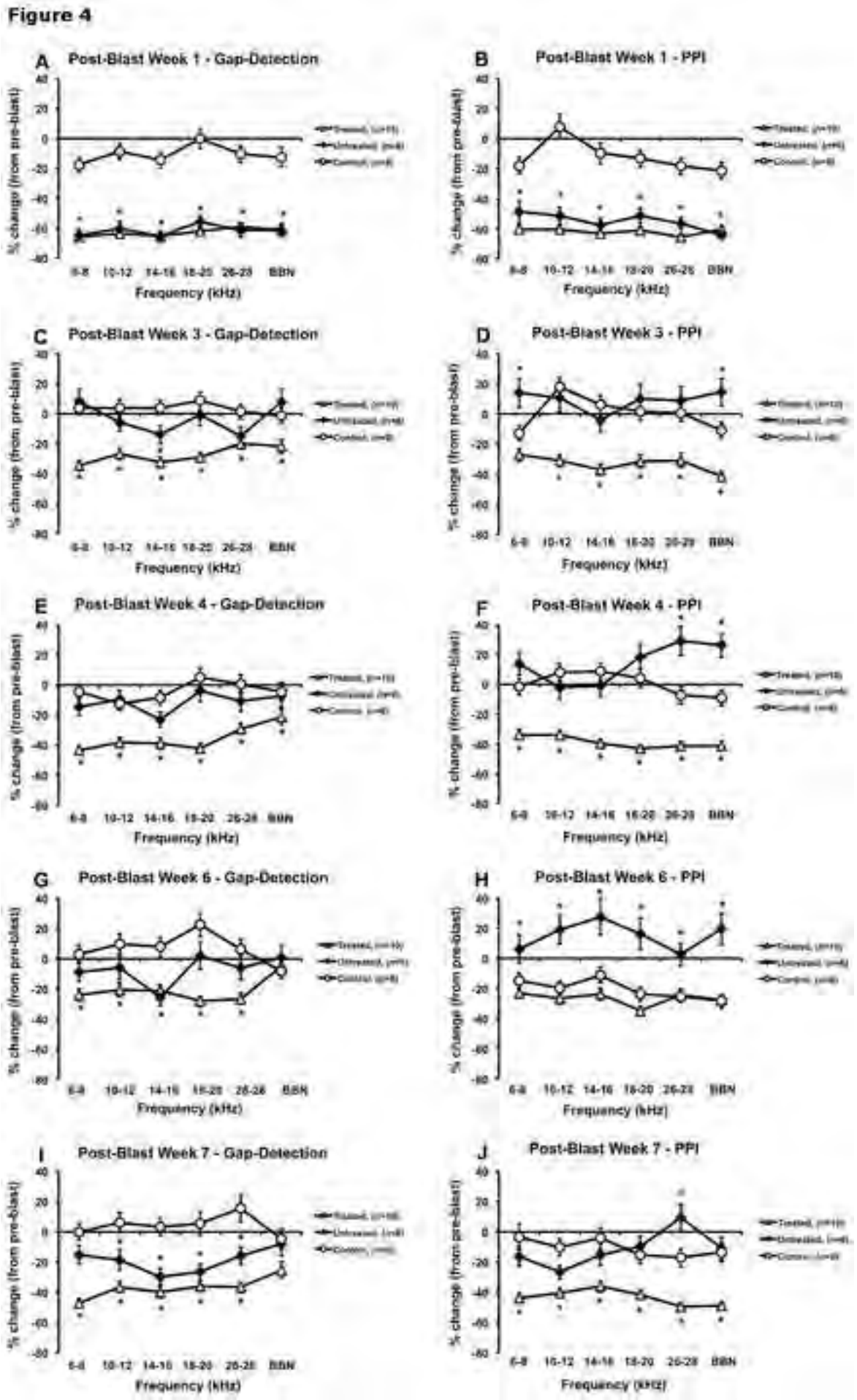

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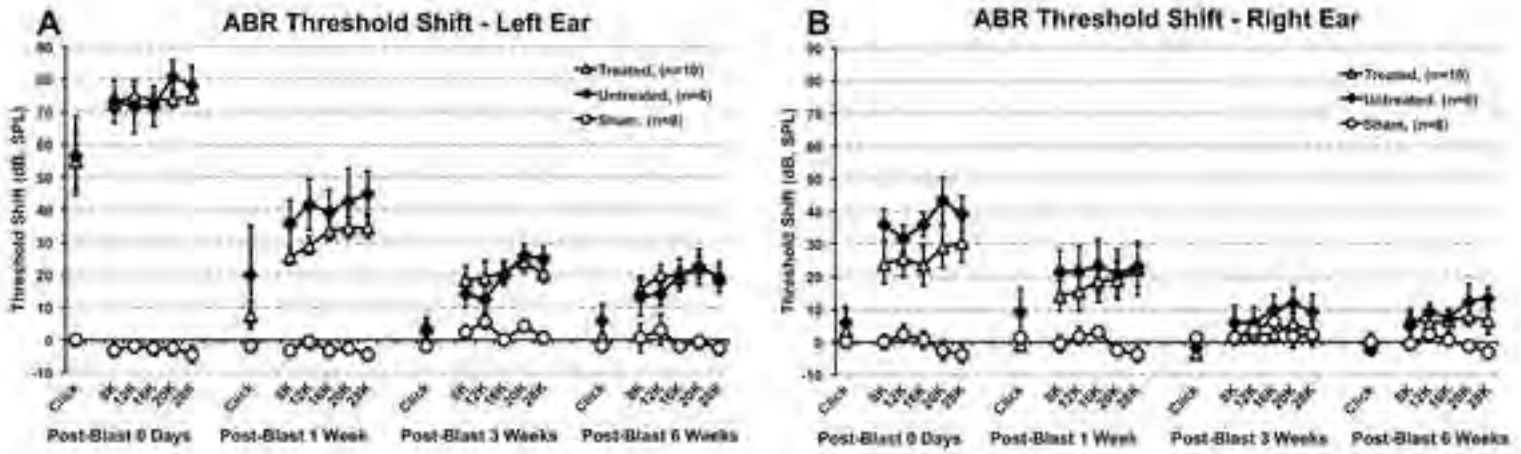

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# Limbic involvement in blast-induced tinnitus, anxiety and related post-traumatic stress disorder in rats

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Running head: Limbic involvement in blast-induced tinnitus
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## Abbreviations:

ACC: anterior cingulate cortex
AMG: amygdala
MEMRI: manganese-enhanced magnetic resonance imaging
NA: nucleus accumbens
PTSD: post-traumatic stress disorder
TBI: traumatic brain injury

## Abstract

Blast-induced tinnitus is a common health condition among soldiers and veterans who experience blast-related trauma. These tinnitus sufferers frequently experience deficits in limbic-associated functioning such as anxiety, memory loss, and depression. It has been suggested that there is strong limbic involvement in the etiology of noise-induced tinnitus, however, it remains unclear how blast invokes the limbic system in tinnitus-related emotional and cognitive problems. In this study, rats were blast-exposed and tested behaviorally for tinnitus, anxiety and spatial cognition using gap detection acoustic startle reflex testing, elevated plus maze and Morris water maze, respectively. Blast-induced neural activity changes in several limbic and paralimbic structures were evaluated using manganese-enhanced magnetic resonance imaging (MEMRI). We found that 8 out of 13 blasted rats developed chronic tinnitus while the other 5 only sustained acute tinnitus, which later remitted. The induced chronic tinnitus was accompanied by increased neuronal activity in the basolateral (deep) and cortical-like (superficial) subdivisions of the amygdaloidal complex as revealed by MEMRI. We also found that blast trauma induced neuronal hyperactivity in the basolateral and cortical-like subdivisions of the amygdala and anterior cingulate cortex, and caused elevated anxiety. Evidence of increased synaptic activity in the amygdala and anterior cingulate cortex core suggests an association between central plasticity, blast-induced tinnitus, and symptoms of post-traumatic stress like anxiety. The possible neural mechanisms were discussed.

***Keywords****: Blast-induced tinnitus, post traumatic stress disorder (PTSD), anxiety, traumatic brain injury (TBI), Amygdala, Rat*

## Introduction

Tinnitus refers to "ringing in the ear" without presence of an external acoustic event. Among many contributing factors, loud noise is the most common inducer of tinnitus (Kaltenbach and Zhang, 2007, Zhang and Guan, 2008, Zhang, 2013). An extreme form of loud noise is blast exposure generated by high-pressure shock waves, which has been recently, reported to induce tinnitus (Darley and Kellman, 2010, Risling et al., 2011). Blast injuries account for over 78% of the combat-related injuries in Operations Iraqi and Enduring Freedom (OIF/OEF) (Owens et al., 2008). Up to 38% of veterans diagnosed with blast-induced traumatic brain injury (TBI) suffer from chronic tinnitus (Lew et al., 2007). According to the "Annual Benefit Report of fiscal year 2012" published by the Veteran Benefits Administration (VBA), there were 971,990 service-connected disability claims related to tinnitus, making tinnitus the most prevalent diagnosis and accounting for 9.7% of all disability claims (VBA, 2013). The annual cost to compensate veterans for tinnitus in the U.S. is nearly $2 billion per year and rising (Liu et al., 2012). In order to reduce patients' suffering and associated costs, there is an urgent need to investigate the mechanisms underlying blast-induced tinnitus, which is currently lacking (Hoare and Hall, 2011, Hoare et al., 2011).

In addition to the bothersome ringing in the ear or head, blast-induced trauma often involves a wide range of pathology in both auditory and non-auditory systems. One common problem is post-traumatic stress disorder (PTSD), an anxiety disorder that is characterized by symptoms of avoidance, intrusiveness and hyperarousal after exposure to stressful and traumatic events (van Wingen et al., 2011). Victims of blast events, especially those who suffered blast-inflicted TBI, are at high risk for developing PTSD (North et al., 1999, Jehel et al., 2003, Shussman et al., 2011, Bass et al., 2012). Moreover, a strong association between tinnitus and

PTSD has been observed, although the underlying mechanisms have yet to be elucidated (Hinton et al., 2006, Fagelson, 2007). A prospective cohort study of OIF/OEF veterans with chronic tinnitus revealed that 34% also had PTSD, with a strong correlation between particularly troublesome tinnitus and PTSD (Fagelson, 2007). Due to the complex nature of PTSD, however, it has been challenging to establish an appropriate animal model. Thus far, several models have been attempted to investigate either some or all aspects of PTSD such as conditioned fear, avoidance learning, fear extinction, and cognitive functioning (Zucker and Smith, 1979, Brennan et al., 2005, Aikins et al., 2011, Corley et al., 2012, Elder et al., 2012, Wang et al., 2012, Matsumoto et al., 2013, Zovkic and Sweatt, 2013). In the current study, we evaluated anxiety and cognition impairment of rats with blast-induced tinnitus to investigate the involvement of certain limbic structures.

Previous animal studies have suggested that neuronal hyperactivity in the amygdala is associated with noise-exposure and salicylate injections previously shown to cause tinnitus (Wallhausser-Franke et al., 2003, Mahlke and Wallhausser-Franke, 2004, Goble et al., 2009). Neuronal expression of proto-oncogene *c-fos* has been linked to neuronal plasticity. Enhanced Fos-like immunoreactivity in the amygdala has been demonstrated in rat, hamster, and gerbil after salicylate injection and/or noise over-exposure (Zhang et al., 2003, Mahlke and Wallhausser-Franke, 2004). Clinical neuroimaging studies have demonstrated abnormal changes in the limbic and paralimbic brain structures of patients with chronic tinnitus. Positron-emission tomography (PET) studies revealed increased cerebral blood flow, presumably due to increased neuronal activity, in the amygdala, hippocampus and anterior cingulate cortex (Lockwood et al., 1998, Mirz et al., 2000, Plewnia et al., 2007, Schecklmann et al., 2013) while functional MRI (fMRI) studies demonstrated hyperactivity in the nucleus accumbens (Leaver et al., 2011), and

MRI voxel-based morphometry (MRI-VEM) revealed decreased grey matter in hippocampus (Landgrebe et al., 2009). Indeed, the role of limbic and paralimbic structures in maintaining tinnitus-related central maladaptive plasticity has gained substantial interest (Rauschecker et al., 2010).

Although both limbic and paralimbic structures appear to play a role in tinnitus, studies have focused mostly on tinnitus resulting from noise-exposure and other etiologies, while blast-induced tinnitus has remained understudied. Thus far, only one study conducted in our lab has focused on blast-induced tinnitus and the associated plastic changes. Anatomically, using diffusion tensor magnetic resonance imaging (DTI-MRI), we found that blast-induced tinnitus was accompanied by axonal rewiring in the inferior colliculus and medial geniculate body (Mao et al., 2012). To further investigate the mechanisms underlying blast-induced tinnitus, along with related PTSD-like symptoms, we set out to investigate the functional changes in several limbic structures, including the amygdala (AMG), anterior cingulate cortex (ACC), and hippocampus, as well as paralimbic structures like the nucleus accumbens(Ueno et al., 2012). Specially, we used manganese enhanced Magnetic Resonance Imaging (MEMRI). In MEMRI, manganese ($Mn^{2+}$) serves as a paramagnetic contrast agent. In the central nervous system, $Mn^{2+}$ enters neurons through voltage-gated calcium channels, Na/Ca exchangers, Na/Mg anti-transporters, and mitochondria calcium channels (Crossgrove and Yokel, 2005). Once it enters the neuron, $Mn^{2+}$ is transported anterogradely along axons and released into the synaptic cleft with glutamine. The intensity of the $Mn^{2+}$ enhanced MR signals are used to represent synaptic activities (Silva and Bock, 2008). That is, the more $Mn^{2+}$ accumulation, the higher the neural activity becomes.

In the current study, we tested a hypothesis that high-pressure blast exposure induces chronic tinnitus associated with PTSD-related traits like anxiety. In addition, we tested another hypothesis that the blast-induced chronic tinnitus and PTSD-like traits are associated with increased neural activity in the limbic and/or paralimbic structures, as evidenced by enhanced $Mn^{2+}$ accumulation.

## Materials and methods

*Animal subjects and Experimental Design*

Thirty Sprague-Dawley rats (male, 60-70 days old at the beginning of experimentation) were purchased from Harlan Laboratories, Inc. Rats were kept on a 12/12h light/dark cycle and were cared for in a federally approved animal vivarium. After auditory brainstem response (ABR), gap detection, and pre-pulse inhibition (PPI) behavioral screening, 19 rats were deemed to have stable behavioral results and normal hearing. They were randomly assigned into two groups. Six rats were used as a sham control group (n=6), and 13 rats were assigned to an experimental (blast) group (n=13). ABR was tested before, immediately after, and 5 weeks after blast exposures. Gap and PPI testing were conducted regularly (at least twice a week) throughout the entire study. Elevated plus maze and Morris water maze were conducted 4 weeks after blast exposure. Twenty-four hours following Morris water maze testing, MEMRI scanning was performed. All rats underwent the same procedures and protocols except that the blast group sustained blast exposure. All experimental protocols and amendments were reviewed and approved by the Animal Investigation Committee at Wayne State University, and all procedures were conducted in accordance with federal animal research guidelines.

*Auditory brainstem responses (ABRs) for testing hearing thresholds*

After becoming acclimated to the vivarium, ABR tests were conducted under isoflurane anesthesia. ABR thresholds in response to clicks and frequency tone burst stimuli were collected at baseline, immediately after blast, and before MEMRI. Each rat was initially anesthetized with a 5% isoflurane/air mixture, which was followed by 2% isoflurane/air for maintenance. Three platinum-coated tungsten electrodes were inserted in the vertex, below the ipsilateral pinna, and in the contralateral temporal muscle for the active, return, and ground electrodes, respectively. Click and tone bursts of 10-msec duration at 8, 12, 16, 20, 28 kHz (0.5-msec rise/fall time) were delivered to the ear canal from an electrostatic speaker. The sound intensity level ranged from 100 to 5 dB SPL in 5 dB SPL decrements. The stimuli were generated by an RX6 multifunction processor and were processed by SigGenRP software (Tucker Davis Technologies system 3). Calibration was achieved using SigCalRP® software. ABR signals were amplified, band-filtered from 0.3 to 3 kHz, notch-filtered at 60 Hz, and averaged 300 to 400 times for click and tone-burst stimuli, respectively.

*Gap detection (Gap) and prepulse inhibition (PPI) acoustic startle behavioral paradigm for diagnosing tinnitus*

A Gap/PPI behavioral paradigm was used to screen tinnitus in rats. The rat was placed into a custom-made conditioning restrainer, which was then placed in a Kinder Scientific sound-attenuation chamber for testing (Kinder Scientific, Poway, CA). The testing was conducted 3 times a week (2-3 days between each session) for 4-6 weeks before blast exposure and twice a week (3-4 days between each session) after the exposure. In the sound-attenuation testing chamber, the startle force of the rat was detected with a piezoelectric transducer attached

underneath the platform. Peak-to-baseline startle responses in Newton were monitored and recorded in real time using Kinder Scientific startle monitor software.

For the gap detection procedure, each rat was tested in the chamber with continuous background noise. The noise consisted of 2 kHz bandpass noise signals at 6-8, 10-12, 14-16, 18-20, or 26-28 kHz, or broadband noise (2-30 kHz), and delivered at 60 dB SPL. The startle stimulus consisted of a 50-msec noise burst delivered at 115 dB SPL. In normal hearing rats, the presence of a 40-msec silent gap beginning at 90-msec before the startle stimulus serves as a strong inhibitory signal to suppress the startle reflex. Inhibition is defined when the startle forces a rat exerts on the platform during the startle-only trials are significantly stronger than the forces it exerts during the gap or PPI trials ($p < 0.05$). Lack of such inhibition as evidenced by high gap/startle-only ratio is referred to "a positive gap test result", which indicates tinnitus or hearing loss. In the PPI paradigm, there was no background noise and the gap was replaced with a 40-msec 60 dB SPL acoustic prepulse consisting of 2 kHz bandpass signals at 6-8, 10-12, 14-16, 18-20, or 26-28 kHz, or broadband noise (2-30 kHz). PPI testing was introduced to further differentiate auditory detection from tinnitus. Lack of inhibition as evidenced by high PPI/startle-only ratios was referred to as "a positive PPI test result", which indicates an auditory detection deficit. A rat was diagnosed with tinnitus if it consistently showed positive gap results and negative PPI results (Turner et al., 2006b, Fitch et al., 2008).

*Blast exposure to induce tinnitus and PTSD-like behavior*

A single blast exposure for the experimental group was conducted using a custom-made shock tube located in the Wayne State University Bioengineering department. (ORA, Inc). To avoid excessive trauma, each rat was placed on a platform 198 inches downstream from the

bursting membrane and 44 inches upstream from the open end of the driven cylinder (Leonardi et al., 2011). After anesthesia with either 4% isoflurane or a ketamine and xylazine mixture (100 mg/kg + 10 mg/kg IP), the right ear of each rat was plugged with a Mack's® earplug and sealed with mineral oil. The rat was wrapped in protective garment and secured on a holder in the driven cylinder. Peak static overpressure of 14 psi (96.5 kPa; 194 dB SPL) and positive phase duration of 2-msec was produced with compressed helium and calibrated Mylar sheets (GE Richards Graphics Supplies Inc., Landsville, PA), the latter of which were placed between the driver and driven cylinder. Blast overpressure burst the Mylar membranes and generated a free-field blast wave similar to that produced by detonating an explosive device (Leonardi et al., 2011). Following blast exposure, each rat was carefully monitored until it regained consciousness.

*Elevated plus maze (EPM) to examine blast-induced anxiety*

Two days before MEMRI scanning, the anxiety level of each rat was measured with elevated plus maze. A camcorder was mounted above the maze on the ceiling to record rats' behaviors in and on the maze. Prior to testing, rats were handled 2 minute/day for 5 days by the same experimenter. On the day of testing, rats were transported to the testing room and acclimated to the dimmed light environment for 4 hours before testing. Each rat was placed in the center of the maze to initiate the test. The rat's behavior was recorded for 5 min. Following the test, the rat was returned to its cage and the maze was cleaned with 70% alcohol followed by air dry. Trials were interspersed with 5-minute-intervals. Each rat was tested for one trial only. Two people rated the videos independently. The scores of the two raters were averaged for each

measure. Anxiety level was determined by calculating the percentage of open-arm entries and time spent on the open-arms (Perrine et al., 2006).

*Morris water maze (MWM) to examine blast-induced cognitive deficits*

At least 24 hours after EPM testing, all rats were tested for spatial learning and memory performance using a one-day Morris water maze protocol as described previously (Pace and Zhang, 2013). The experiment took place in a square room (9 $m^2$) with a painting for visual reference. The rat swam in a circular fiberglass pool with a diameter of 183 cm. The pool was filled with water and opacified with black tempera paint. A hidden platform (11 cm in diameter) was placed inside the pool one inch below the water surface (Morris, 1984). Rats were towel dried between each swimming trial. The swimming trajectory of each rat was recorded using a digital camcorder mounted on the ceiling. Rats were tracked and measured by Ethovision software (Noldus Information Technology, Inc.). Latency was defined as the amount of the time the rat spent to find the platform in each trial. The latency, swimming velocity, and time spent in each of the 4 zones were calculated for each trial.

*Spatial acquisition task*: In this task, rats were trained to use extra-maze cues to find the hidden platform. The submerged platform (hidden) was placed in southeast quadrant (Zone 4) of the tank. In this task, each rat swam 12 total trials, which were organized into three blocks of 4 trials each. The rat entered the pool at one of 4 random starting positions (N, S, E, W). To alleviate potential anxiety, the rat was gently placed into the water facing the pool wall. If the rat failed to locate the hidden platform within 60 seconds, it was taken out of the water and placed on the platform for approximately 3 seconds. All rats rested in their cages for 30 minutes

between blocks. Trial latencies were calculated to assess spatial learning, while velocity represented the degree of mobility.

*Probe trial*s: Thirty minutes following the third block of the spatial acquisition task, rats were tested with one probe trial. During the probe trial, the platform was removed and a rat was placed into the pool for 60 seconds. The amount of time spent and the number of entries the rat made into the former platform position (Zone 4) was used to reflect spatial memory.

This compressed protocol has been validated as a sufficient tool to assess spatial reference memory and spatial learning elsewhere (Feng et al., 2012, Zhang et al., 2012)(Pace and Zhang, 2013). A 1-day protocol was preferable to a 3- or a 5-day protocol since tinnitus perception may fluctuate across testing days and simultaneously assessing tinnitus and spatial cognition would be too stressful for animals.

*MEMRI imaging*

Five weeks after the blast exposure, all rats were scanned with a 7.0 T Siemens ClinScan MRI scanner (Siemens Medical Solutions USA, Inc. Malvern, PA). Rats were injected with $MnCl_2$ (67 mg/kg body weight) intraperitoneally. After the injection, rats were placed in a soundproof room for 8 hours to allow absorption and neuronal uptake of manganese. Accumulative uptake of manganese for 8 hours is adequate for functional imaging, and utilization of anesthesia during scanning does not affect manganese uptake (Lee et al., 2005, Bissig and Berkowitz, 2009). Before scanning, rats were induced with 4% isoflurane/air mixture in an induction chamber. Subsequently, anesthesia was maintained during scanning with a 2% isoflurane/air mixture via a commercially made MRI compatible nose cone. During scanning, the rat was placed on a heated re-circulating water pad to maintain core body temperature. A

whole-body transmit-only coil and a 4-element Burker mouse-brain receive-only surface coil placed dorsal to the rat's head were used for scanning. T1-weighted and 3D gradient-echo images were acquired. Individual images were acquired with 2 sets of MPRAGE (TR: 2500 msec, TE: 3 msec, TI: 1500 msec) and PDGE (TR: 1000 msec, TE: 3 msec) sequences. The flip angle was 3°. The field of view was a 25 X 25 section of space, which was 192 pixel X 192 pixel in image resolution. The thickness of the slice between adjacent images was 0.26 mm. At the conclusion of the scanning, the rat was monitored until regaining consciousness on a heated pad. Fully awake animals were then returned to their home cages. They were closely monitored for another 8 hours after the scan until their return to the vivarium.

Images were processed with R (v2.12.1, http://www.r-project.org) scripts developed in-house by Dr. D. Bissig. The addition of two sets of MPRAGE images was divided by the addition of two sets of PDGE images to mitigate the intensity field bias. The corrected images were used for analysis. Regions of interest (ROIs) were manually drawn using MRIcro v.1.40 with gingerly referencing the Paxinos and Watson ($4^{th}$ Edition; 1998) rat brain atlas (Figure 1). Average signal intensity of ROIs was obtained with MRIcro. ROI signal intensities (SI) were normalized with that of adjacent noise ($SNR = 1000*SI_{\_ROI}/SI_{\_noise}$). Signal-to-noise normalization has been utilized to study auditory structures with MEMRI. The following structures were included for analysis: the amygdala (AMG), anterior cingulate cortex (ACC), hippocampus, and nucleus accumbens (Acb). Two blinded experimenters independently assessed the data using the same criteria. All image sets were randomized. The two sets of results were analyzed for inter-rater variability using Pearson correlation analysis. The Pearson coefficients were over 60% for all structures.

**Figure 1.**

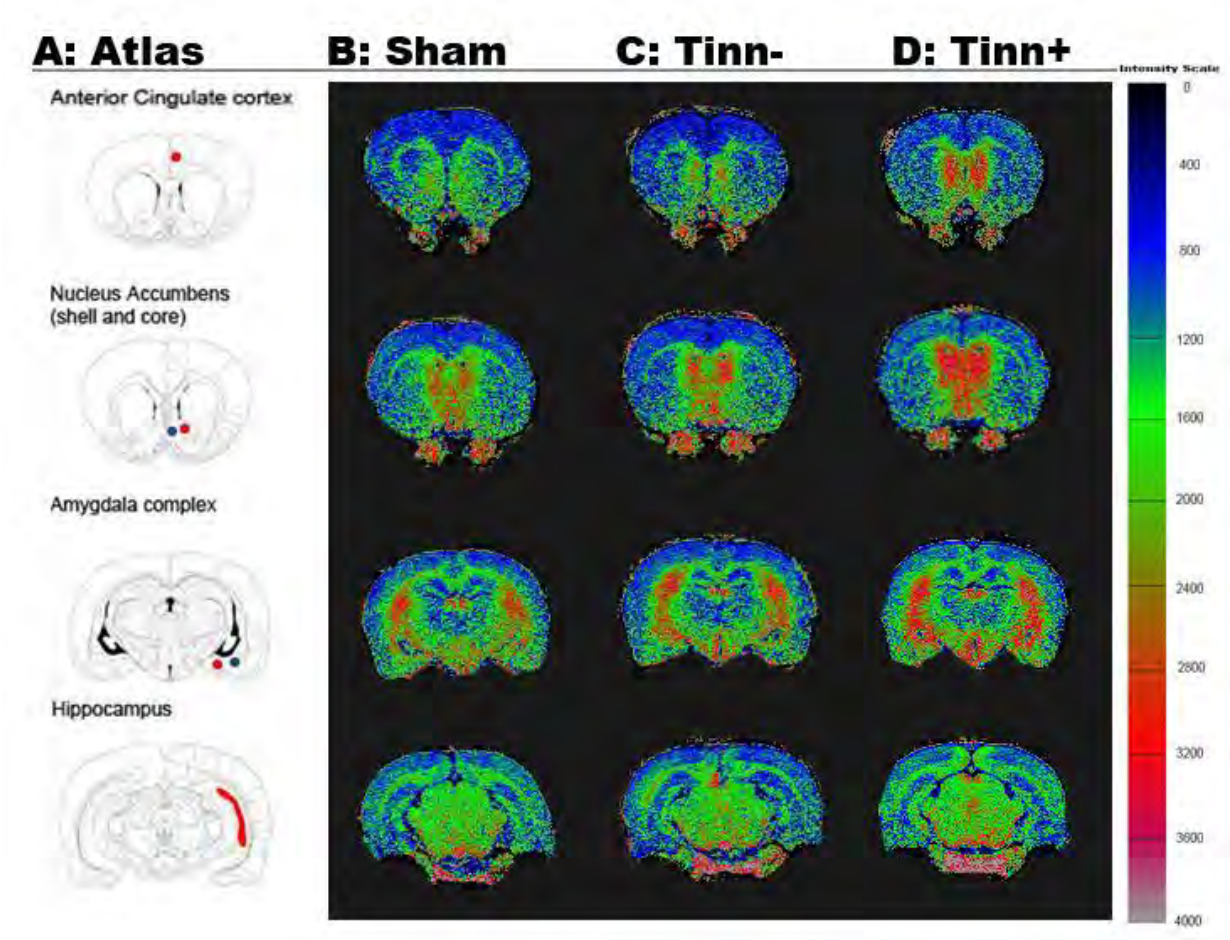


Figure 1. The illustration of regions of interest (ROI). Column A: Rat brain atlas. From top to bottom: anterior cingulate cortex (red dot), nucleus accumbens (red dot is core, and blue dot is shell), amygdala (red dot is superficial group, and blue dot is deep group), and hippocampus (red line is CA1/CA2 regions). Column B through D are representative images from the sham, tinnitus negative, and tinnitus positive groups.

*Procedures to measure signal-to-noise ratio (SNR) values for different structures*

*Measurement of SNR in the AMG, ACC, and Acb (Figure 1)*. To accurately obtain the SNR values, we divided the amygdala complex into three subdivisions according to their distinct functions: centromedial nuclei ($AMG_C$), the superficial or cortical-like nuclei ($AMG_S$), and deep or basolateral nuclei complex ($AMG_D$). Spherical 3D ROIs were used to characterize the amygdala (AMG), anterior cingulate cortex (ACC), and nucleus accumbens (Acb) (radius: 260 micron). The ROIs were placed at the rostrocaudal center of each anatomical region using the coronal profile, excluding a buffer (≥1 voxel wide, depending on the ROI) at borders with neighboring brain regions and tissues (e.g. choroid plexus). Appropriate ROI placement was confirmed in parasagittal and transverse profile views.

*Measurement of SNR in the hippocampus (Figure 2)*. Intensity of hippocampus was obtained with ImageJ. Three consecutive slices were used on each side. The first slice was caudal to the slice where the dentate gyrus became evident. Four lines were drawn on each side so that 1) they were perpendicular to the curvature of the forceps of the major corpus callosum; 2) the midpoint of the line aligned with the border of the forceps major, and; 3) the direction of the line was toward the center of the parenchyma (Figure 2A). The lines were drawn on the PDGE image set then copied to the MPRAGE image set on the same corresponding coronal slice. A gray value linear plot was generated from each line copied to the MPRAGE image (Figure 2, B). The x-axis represents the distance the line "travels", and y-axis represents the signal intensity along the line in the form of a "gray value" The peak whose distance just passed mid-point represented the position of hippocampal CA1 and CA2 neurons. The sum of the peak values on one side of the hemisphere was used to represent signal intensity of each hippocampal ROI.

**Figure 2.**

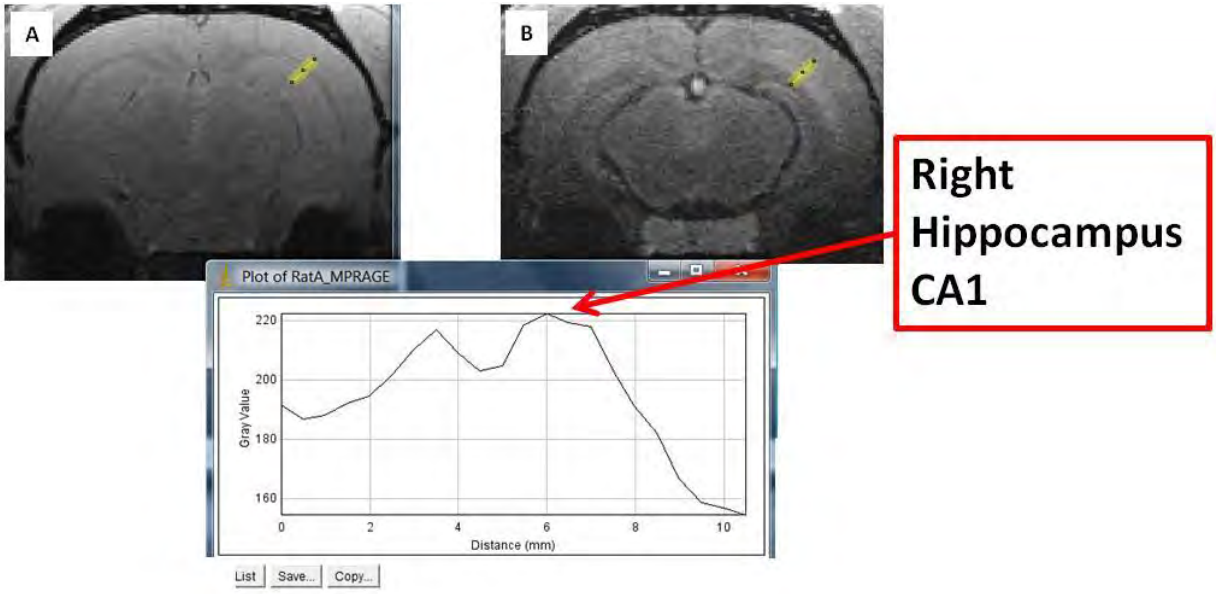


Figure 2. Illustration of the method to obtain signal intensity values for CA1/CA2 regions of the hippocampus. A: picture of a PDGE image. The yellow line indicates the ROI. B: ROI is copied to MPRAGE image.

*Data analysis.*

ABR thresholds were compared between groups by subtracting the pre-blast threshold from the post-blast threshold. Thresholds were considered to be the lowest sound intensity at which a distinct portion of the biological ABR waveform remained visible.

For testing tinnitus, gap-detection data were divided into ratios as described previously (Zhang et al., 2011, Luo et al., 2012, Mao et al., 2012, Shekhawat et al., 2013). Briefly, for each frequency or BBN, the startle force in response to the GAP condition was divided by the mean response to the associated startle only condition, which generated a ratio value between 0 and 1. A value close to 0 would indicate strong suppression of the startle reflex in response to silent

gaps, and thus healthy status, whereas a value close to 1 would signify little suppression in response to the gap, potentially indicating tinnitus (Turner et al., 2006a, Yang et al., 2007, Wang et al., 2009, Kraus et al., 2011, Longenecker and Galazyuk, 2011, Nowotny et al., 2011, Zhang et al., 2011, Luo et al., 2012, Mao et al., 2012). PPI data were analyzed in the same manner, and upward or downward changes in PPI data would indicate auditory detection loss or improvement, respectively. Blasted rats that exhibited elevated GAP ratios and unaffected PPI ratios prior to MEMRI scanning were placed into the tinnitus positive group while those that did not display elevated ratios were placed into the tinnitus negative group. Sham control rats were similarly assessed to ensure that they did not spontaneously develop tinnitus during the course of experiments.

In EPM testing, anxiety was assessed by calculating the percent of entries into and time spent in the open-arms. Compared to controls, reduced entries and time in the open-arm would indicate higher anxiety, while increased entries and time suggests less anxiety (Nobre and Brandao, 2011).

Morris water maze data were compared between the three groups to examine the effects of acoustic trauma and tinnitus on spatial learning and memory. Spatial learning was evaluated by the escape latency trials, whereas spatial memory was gauged in the probe trial by the amount of time spent and entries into the target zone. Longer escape latencies and a lower affinity for the target zone would suggest impaired spatial learning and memory.

To analyze MEMRI data, two experimenters independently rated images to calculate the differences in SNR values for each ROI. Higher values would indicate greater neuronal activity. To confirm inter-rater reliability, Pearson correlation analysis was conducted. The statistical

analysis was performed with a commercial software package (SPSS 20.0 for windows, Chicago, IL).

For all pre- and post-exposure and between-group comparisons, one-way ANOVA was performed with a post-hoc Bonferroni test to adjust alpha values. For pre- and post-exposure comparisons in gap-detection and PPI data for individual rats, t-test assuming unequal variances was used. $P < 0.05$ was considered as indication of statistical significance.

## Results

*ABR data*

Compared to baseline click and tone-burst hearing thresholds, hearing thresholds recovered at 5 weeks post-blast prior to MEMRI scanning. No significant differences were found between exposed and non-exposed ears and between blast and the sham groups ($p > 0.05$) (Figure 3).

## Figure 3.

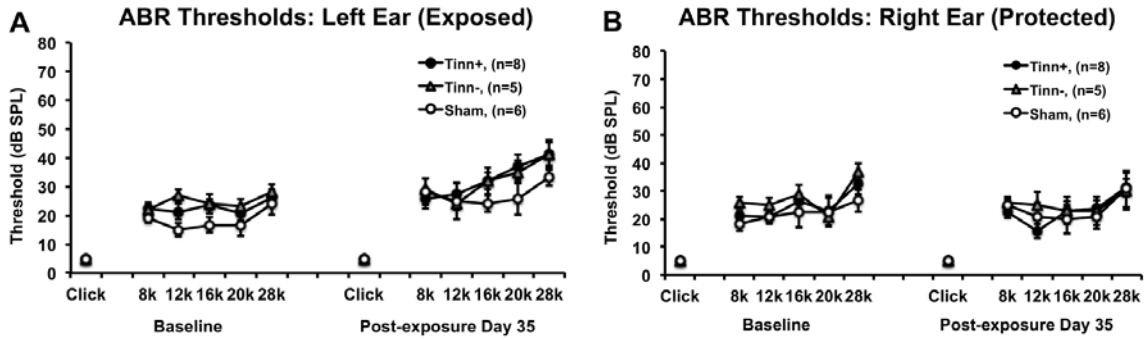


Figure 3. Auditory brainstem response (ABR) thresholds from the exposed left ear (A) and protected right ear (B). There were no significant differences between the three groups in either ear at baseline or at 35 days post-blast. Error bars represent SEM.

*Behavioral data*

Blasted rats with elevated gap-detection ratios and unaffected PPI ratios prior to MEMRI scanning were placed into the tinnitus positive group while the others were placed into the tinnitus negative group. As can be seen in Figure 4 (A &B), the tinnitus positive group ("Tinn+", n=8, Figure 4A & 4B) exhibited robust evidence of tinnitus at 28 kHz five weeks after blast exposure ($p < 0.05$) whereas the tinnitus negative group ("Tinn-", n=5, Figure 4C & 4D) and the sham group ("Sham", n=6, Figure 4E & 4F) did not ($p > 0.05$).

**Figure 4.**

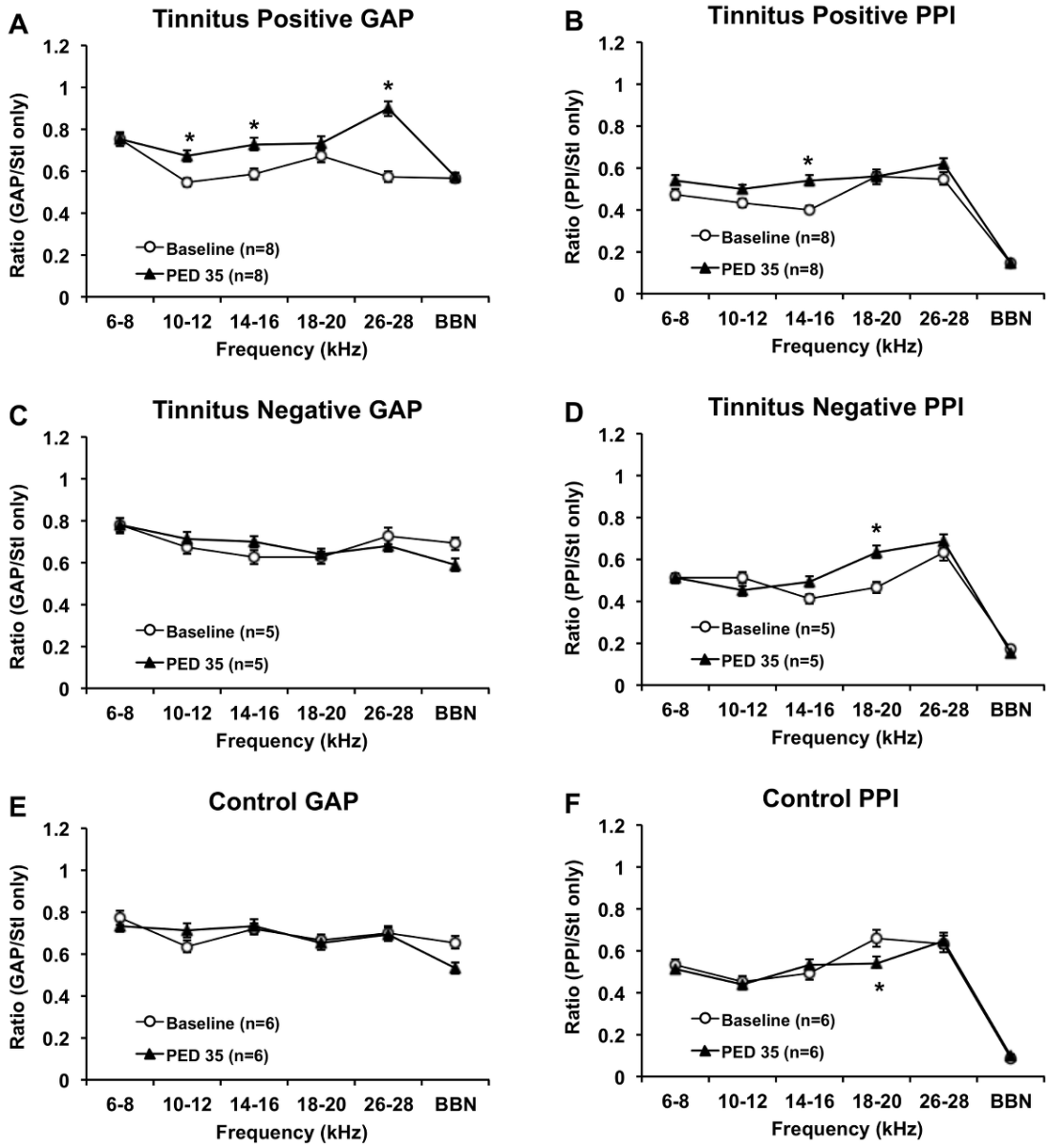


Figure 4. GAP and PPI ratios for the tinnitus positive, tinnitus negative and sham groups. Gap detection data revealed that the tinnitus positive group exhibited robust behavioral evidence of 12, 16, and 28 kHz tinnitus at 35 days after exposure (PED35) compared to baseline (BL) [significant increase in ratio ($p<0.05$) (A)], while the tinnitus negative and sham groups exhibited no evidence of tinnitus [no significant increase in ratio ($p>0.05$), (C)&(E)]. Prepulse inhibition data showed auditory detection deficits at 35 days after blast exposure at 16 kHz in the tinnitus positive group ($p<0.05$) (B); (2) at 20 kHz in the tinnitus negative group ($p<0.05$) (D) but a significant decrease in PPI ratio at 20 kHz ($p<0.05$) (F)]. Error bars represent SEM.

When all the blasted rats were grouped together (n=13), the average gap detection and PPI data were compared against those of the sham group. As seen in Figure 5, the blasted group exhibited strong behavioral evidence of tinnitus at 26~28 kHz (Figure 5A), and no significant change in PPI ratios (p = 0.63) (Figure 5B).

## Figure 5.

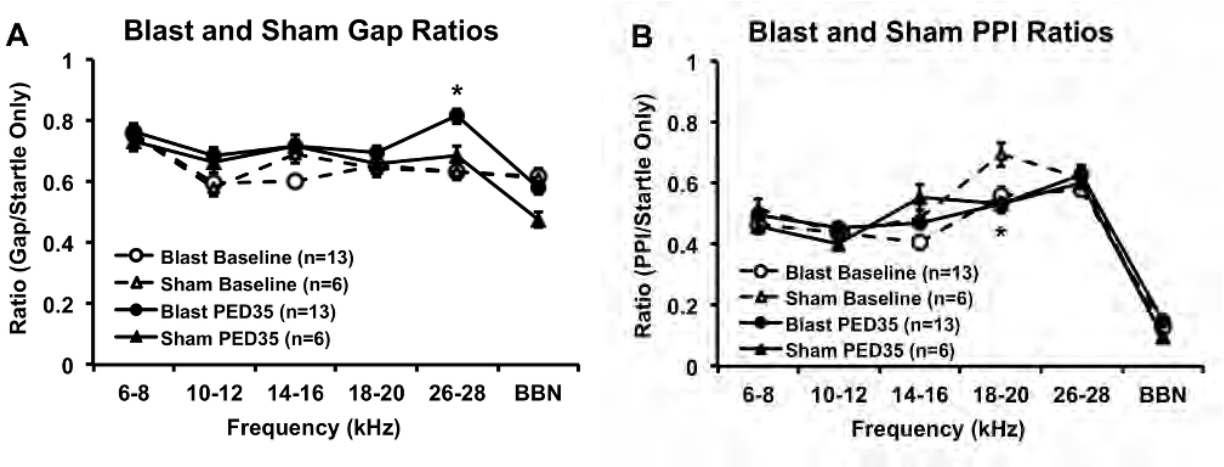


Figure 5. GAP and PPI ratios for the blast and sham groups. Gap detection data showed that (1) 35 days after the blast exposure (PED35), blast group (n=13) exhibited strong behavioral evidence of tinnitus (BL) at 28 kHz [significant increase in ratio ($p<0.05$), (A)]; and (2) 35 days after blast exposure, the sham group did not exhibit evidence of tinnitus [no significant increase in ratio ($p>0.05$) <0.8, (A)]. Prepulse inhibition data showed no behavioral evidence of auditory detection deficits 35 days after blast exposure: [(1) 35 days after the blast exposure, the blast group did not exhibit significant increase in PPI ratio at any frequency bands ($p<0.05$) and (B); (2) 35 days after the blast exposure, the sham group exhibited significant decrease in PPI ratio at 20 kHz ($p<0.05$) (B)]. Error bars represent SEM.

*Elevated plus maze testing results*

Rats in the sham group committed an average of 32.6±7.7% of open-arm entries and spent an average time of 26.2±6.8% percent on the open arms. Rats in the blasted group committed an average of 9.8±4.7% of open-arm entries and spent an average time of 4.8±2.8% in the open arms. Compared to blasted rats, rats in sham group spent significantly more time in the open arms ($p<0.05$) and made significantly more entries into the open arms ($p<0.05$) (Figure 6). This clearly demonstrated that blast exposure induced significant anxiety.

## Figure 6.

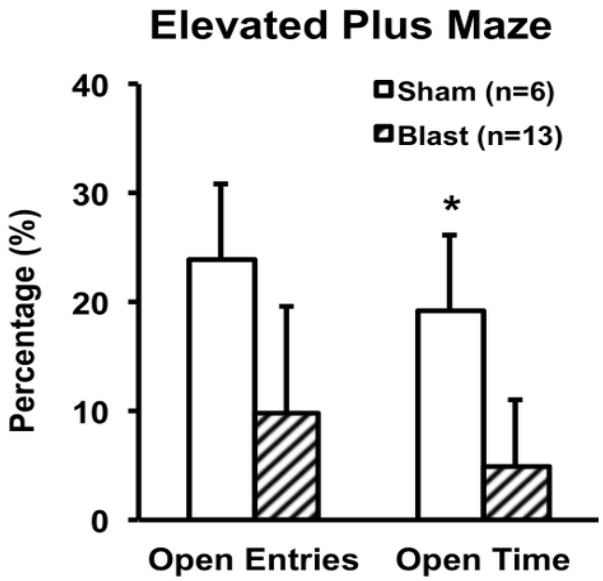


Figure 6. Percent of open-arm entries and open-arm time in the elevated plus maze. Compared to the sham group, blasted rats made significantly less entries to the open-arms and spent significantly more time on the open-arms ($p<0.05$), indicating significantly higher anxiety levels. Error bars represent SEM.

*Morris water maze test results*

*Spatial task acquisition*. Three blocks comprised of four trials apiece served as the spatial acquisition task trials (Figure 7A & 7B). There was no significant difference in the latency and velocity among the three groups in blocks 1 through 3 ($p>0.05$), indicating there was no difference in the spatial learning among the three groups.

*Probe trial acquisition*. No significant differences in target zone entries or time were observed between groups in the probe trial ($p>0.05$, Figure 7C). These results mirror our recent findings that not all tinnitus positive animals develop cognitive impairment (Pace and Zhang, 2013).

## Figure 7.

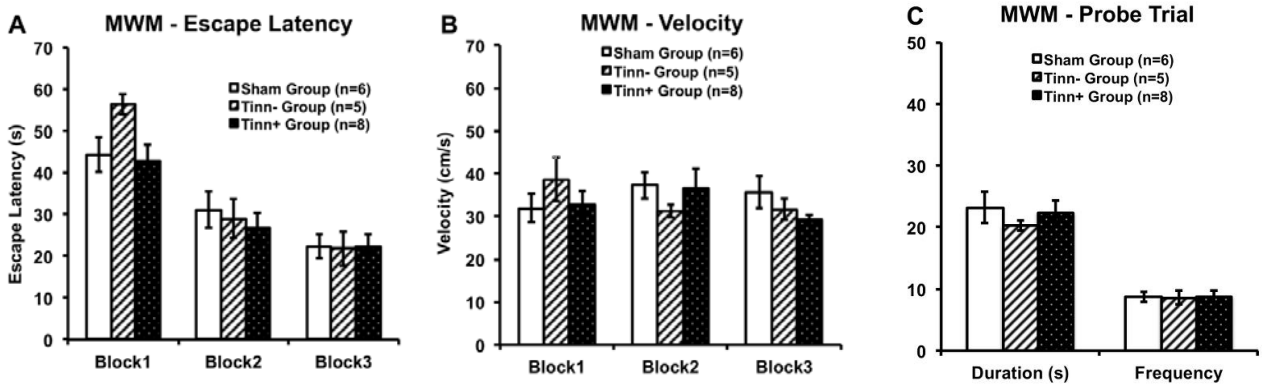


Figure 7. Morris water maze escape latency, velocity, and probe trial data. No significance differences were seen among the tinnitus (+), tinnitus (-), and sham group in escape latency (A), velocity (B), or probe trial target zone entries or time (C) ($p>0.05$), indicating similar spatial learning and memory across groups. Error bars represent SEM.

*MEMRI data*

From the time of injection to the time of MRI scanning, the average time of manganese uptake for the sham and blast groups were 648.2±3.2 min and 640.3±2.1 min, respectively. The differences of time for manganese uptake between groups were not statistically significant (*p>0.05*). Table 1 listed the signal-to-noise ratio (SNR) values for the regions of interest. Compared to the sham group, the tinnitus positive group demonstrated higher $Mn^{2+}$ uptake in the bilateral amygdaloidal complex, specifically contralateral cortico-like (superficial) groups and ipsilateral basolateral group of amygdaloidal complex ($p < 0.05$). There was no difference in $Mn^{2+}$ uptake between the sham and tinnitus (-) groups ($p > 0.05$). Figure 8 shows the data comparing bilateral ROI SNRs among the three groups.

**Table 1. The signal-to-noise ratios of limbic and paralimbic structures.**

| Limbic and Paralimbic Structures | | | | Mean SNR | SD of SNR | *sig. (p)* | | *Pearson's correlation coefficient* |
|---|---|---|---|---|---|---|---|---|
| Amygdaloidal Complex | **AMG_S (superficial group)** | Left | Tinn+ | 0.664 | 0.033 | Tinn+ vs. sham | *0.067* | *0.678* |
| | | | Tinn- | 0.649 | 0.051 | Tinn- vs. sham | *0.339* | |
| | | | Sham | 0.605 | 0.049 | Tinn+ vs. Tinn- | *1* | |
| | | **Right** | Tinn+ | 0.668 | 0.053 | **Tinn+ vs. sham** | ***0.020*** | *0.706* |
| | | | Tinn- | 0.657 | 0.050 | Tinn- vs. sham | *0.081* | |
| | | | Sham | 0.578 | 0.058 | Tinn+ vs. Tinn- | *1* | |
| | **AMG_D (deep group)** | **Left** | Tinn+ | 0.716 | 0.042 | **Tinn+ vs. sham** | ***0.015*** | *0.456* |
| | | | Tinn- | 0.676 | 0.038 | Tinn- vs. sham | *0.713* | |
| | | | Sham | 0.647 | 0.037 | Tinn+ vs. Tinn- | *0.280* | |
| | | Right | Tinn+ | 0.686 | 0.045 | Tinn+ vs. sham | *0.050* | *0.324* |
| | | | Tinn- | 0.664 | 0.061 | Tinn- vs. sham | *0.353* | |
| | | | Sham | 0.615 | 0.045 | Tinn+ vs. Tinn- | *1* | |
| | AMG_C (central group) | Left | Tinn+ | 0.673 | 0.042 | Tinn+ vs. sham | *0.215* | *0.726* |
| | | | Tinn- | 0.627 | 0.039 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.629 | 0.043 | Tinn+ vs. Tinn- | *0.222* | |
| | | Right | Tinn+ | 0.665 | 0.057 | Tinn+ vs. sham | *0.595* | *0.718* |
| | | | Tinn- | 0.670 | 0.080 | Tinn- vs. sham | *0.591* | |
| | | | Sham | 0.622 | 0.043 | Tinn+ vs. Tinn- | *1* | |
| Nucleus Accumbens | Acb_C (core) | Left | Tinn+ | 0.697 | 0.061 | Tinn+ vs. sham | *0.082* | *0.505* |
| | | | Tinn- | 0.650 | 0.040 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.629 | 0.045 | Tinn+ vs. Tinn- | *0.416* | |
| | | Right | Tinn+ | 0.691 | 0.060 | Tinn+ vs. sham | *0.156* | *0.492* |
| | | | Tinn- | 0.666 | 0.038 | Tinn- vs. sham | *0.823* | |
| | | | Sham | 0.627 | 0.063 | Tinn+ vs. Tinn- | *1* | |
| | Acb_S (shell) | Left | Tinn+ | 0.681 | 0.050 | Tinn+ vs. sham | *0.388* | *0.706* |
| | | | Tinn- | 0.649 | 0.020 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.642 | 0.052 | Tinn+ vs. Tinn- | *0.717* | |
| | | Right | Tinn+ | 0.679 | 0.047 | Tinn+ vs. sham | *0.894* | *0.603* |
| | | | Tinn- | 0.622 | 0.087 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.643 | 0.053 | Tinn+ vs. Tinn- | *0.366* | |
| ACC (anterior cingulate cortex) | | Left | Tinn+ | 0.478 | 0.023 | Tinn+ vs. sham | *0.593* | *0.638* |
| | | | Tinn- | 0.482 | 0.030 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.461 | 0.015 | Tinn+ vs. Tinn- | *1* | |
| | | Right | Tinn+ | 0.481 | 0.022 | Tinn+ vs. sham | *0.183* | *0.671* |
| | | | Tinn- | 0.481 | 0.014 | Tinn- vs. sham | *0.273* | |
| | | | Sham | 0.460 | 0.020 | Tinn+ vs. Tinn- | *1* | |
| Hippocampus | | Left | Tinn+ | 0.623 | 0.079 | Tinn+ vs. sham | *1* | *N/A* |
| | | | Tinn- | 0.652 | 0.138 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.589 | 0.070 | Tinn+ vs. Tinn- | *1* | |
| | | Right | Tinn+ | 0.620 | 0.060 | Tinn+ vs. sham | *0.552* | *N/A* |
| | | | Tinn- | 0.599 | 0.128 | Tinn- vs. sham | *1* | |
| | | | Sham | 0.557 | 0.070 | Tinn+ vs. Tinn- | *1* | |

Note: Compared to the sham group, the tinnitus (+) group showed higher $Mn^{2+}$ uptake in ipsilateral deep group of amygdala and contralateral superficial group of amygdala ($p<0.05$); and no differences were found between the tinnitus (-) and sham groups ($p>0.05$).

**Figure 8.**

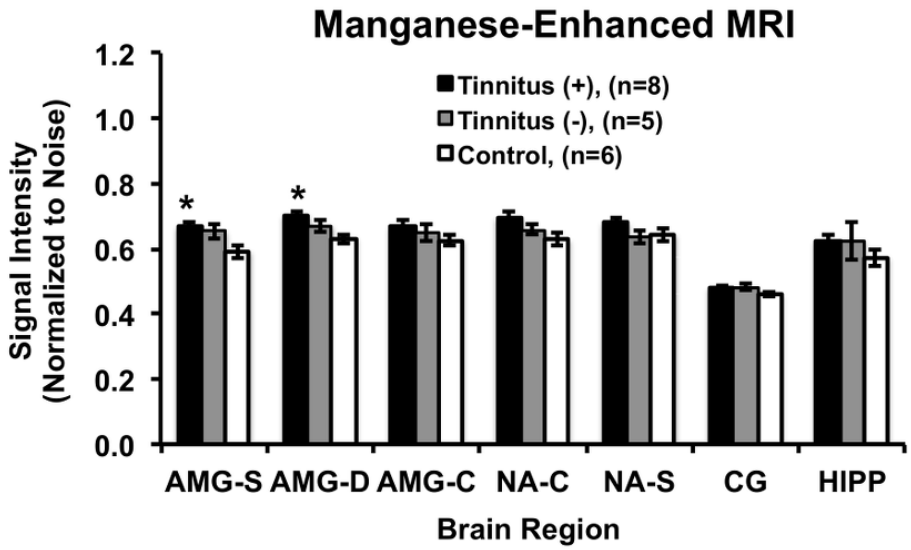


Figure 8. The average bilateral signal-to-noise ratios of limbic and paralimbic structures. Compared to the sham group, the tinnitus (+) group showed higher $Mn^{2+}$ uptake in the superficial (AMG-S and deep subdivisions (AMG-D) of the amygdaloidal complex ($p<0.05$); no difference were found between the tinnitus (-) and sham group ($p>0.05$).

When the tinnitus (+) and tinnitus (-) groups were combined into the blast group (n=13), the resulting group averages of SNRs were compared with those of the sham group. We found that, compared to the sham group, the blast group demonstrated higher manganese uptake in bilateral superficial and deep groups of the amygdaloidal complex and anterior cingulate cortex ($p < 0.05$), as seen in Figure 9.

**Figure 9.**

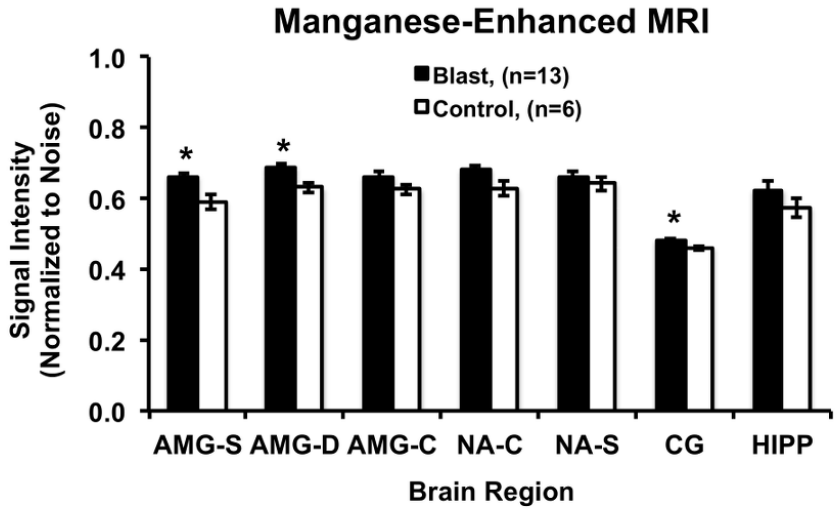


Figure 9. The average bilateral signal-to-noise ratios of limbic and paralimbic structures. Compared to the sham group, the blast group showed higher $Mn^{2+}$ uptake in the superficial (AMG-S) and deep (AMG-D) subdivisions of the amygdaloidal complex and anterior cingulate cortex ($p<0.05$).

## Discussion

The results of the current study demonstrated that a single blast exposure at 14-psi induced robust chronic behavioral evidence of tinnitus in 8 out of 13 rats. The results support our previous report that blast exposure induces behavioral evidence of tinnitus (Mao et al., 2012). The blast exposure also induced significant increases in anxiety level. Blasted rats showed hyperactivity in the amygdala and anterior cingulate cortex. However, rats with blast-induced tinnitus showed hyperactivity in the deep and superficial subdivisions of the amygdaloidal complex. The results are in line with the clinical observations that blast-induced tinnitus is often

accompanied by PTSD symptoms. However, our results showed that rats with blast-induced tinnitus did not experience significant cognitive impairment, paralleling results from our recent report showing that noise-induced tinnitus was not associated with cognitive impairment (Pace and Zhang, 2013). These data indicate that the behavioral models used to optimally test anxiety and cognitive impairment need to be further explored. Nevertheless, the current study provided evidence of strong involvement of both limbic and paralimbic structures in the etiology of blast-induced tinnitus and associated PTSD-like symptomology.

*The involvement of the amygdala in tinnitus-related stress and anxiety*

Deep within the temporal lobe, the amygdala has been shown to be the pivotal component in the network that underpins fear-related responses. The amygdala assigns emotional significance and yields responses to external stimuli (Pitkanen et al., 2000). Based on its cytoarchitectonic and connectional characteristics, the amygdala, a.k.a. amygdaloidal complex, is commonly divided into four groups: the deep or basolateral group (AMG_D), superficial or cortical-like group (AMG_S), centromedial group (AMG_C), and amygdalohippocampal area (AHA) (Sah and Lopez De Armentia, 2003). The amygdaloidal complex network reciprocally interacts with other brain regions such as the prefrontal cortex, anterior cingulate cortex and hippocampus. The basolateral group of the amygdala receives afferent signals from prefrontal cortex, which are then projected to central nuclei, hippocampus, and nucleus accumbens among others; the centromedial group then relays cholinergic projections from and to striatum, hypothalamus, and basal forebrain (Bigl et al., 1982; .Woolf et al., 1986).

The amygdala is a key structure in the mechanism of several stress-related disorders. In the case of chronic tinnitus, the tinnitus triggers emotional stress; the amygdala consolidates tinnitus-

induced stress; then it facilitates the release of stress hormones through hypothalamus-pituitary-adrenal (HPA) axis, and the consolidation of tinnitus-related memory through the hippocampus (Kraus and Canlon, 2012). Based on this notion, treatments that targeted some limbic structures were developed and proven to be efficacious. In one such clinical trial, when a GABA agonist (amobarbital) was unilaterally injected into anterior choroidal artery, which supplies amygdalohippocampal region, the contralateral tinnitus was suppressed (De Ridder et al., 2006). In the case of PTSD, the amygdala serves as the hub of fear conditioning and plays vital roles in mediating PTSD. Closely related, fear conditioning and PTSD share similar neural circuitry and molecular mechanisms; and the animal models for studying fear conditioning have been used to study the mechanisms of PTSD (Chen et al., 2011). Auditory fear conditioning, a subtype of fear conditioning, requires the participation of two amygdala-associated pathways, which are thalamo-amygdala pathway and thalamo-cortico-amygdala pathway. The centromedial group of amygdala is the center of thalamo-amygdala pathway; and the basolateral group of amygdala is the center of thalamo-cortico-amygdala pathway (Romanski and LeDoux, 1992). Studies from fear conditioning have provided compelling evidence that the acquisition and storage of fear-related memory is mediated by the lateral nucleus of the amygdala through NMDA receptor-mediated synaptic plasticity (Yang et al., 2012).

Based on Elder's blast-induced PTSD rat model (Elder et al., 2012), we conducted elevated plus maze, one of the many cognitive/psychological tests in Elder's protocol, to test anxiety level. Our blasted rats showed significantly higher anxiety compared to the sham group rats. In Elder's model, rats were exposed to three blast exposures at 10.8-psi under general anesthesia, and the rats exhibited increased anxiety, enhanced contextual fear conditioning, and an abnormal response in a predator scent assay, along with an elevated level of protein stathmin 1 in the

amygdala. In contrast to his study, we subjected rats to a single 14-psi blast. Even though we did not test open field activity, predator scent, or contextual fear conditioning; our results mirrored Elder's findings of elevated anxiety level and hyperactivity in amygdala. It is highly probable that the blasted rats in the current study exhibited chronic PTSD-related traits. The fact that we found enhanced synaptic activity in the form of elevated $Mn^{2+}$ neuronal uptake in the basolateral (deep) and cortical-like (superficial) but not centromedial subdivision indicates that the thalamo-cortico-amygdala pathway may be a key component of the mechanistic network underpinning blast-induced tinnitus and associated PTSD-like symptoms.

*The participation of the anterior cingulate cortex in tinnitus-related anxiety*

The anterior cingulate cortex (ACC) plays a vital role in both cognitive and emotional processes. It has extensive reciprocal connections with the prefrontal cortex, and the latter is the key player in the cognitive network whose functions include motivation, error detection, complex motor control, and working memory; furthermore, it networks with the amygdala, nucleus accumbens, hypothalamus, insula, hippocampus, and orbitofrontal cortex to participate in the regulation of emotional and stress responses (Bush et al., 2000, Lazar et al., 2000). Based on its anatomy and cytoarchitecture, the ACC can be divided into the ventral portion and dorsal portion. The ventral portion of the ACC involves in the regulation of the emotional and stress responses (Etkin et al., 2006). Hyperactivity in the ACC has been associated with chronic tinnitus (Mirz et al., 2000, Plewnia et al., 2007). Our findings that blast-induced tinnitus was associated with hyperactivity in the amygdala and ACC have resonated with these previous findings.

"ACC-amygdala coupling" refers to the connectivity between the amygdala and the anterior cingulate cortex. In the thalamo-cortico-amygdala pathway, fear-related sensory signals pass through the thalamus and then relay to an elaborate network of sensory cortices before reaching the lateral nucleus of the amygdala for explicit processing (Das et al., 2005). This pathway is regulated by the anterior cingulate cortex in a glucocorticoid-dependent manner (Stutzmann et al., 1998). Different regions of the ACC exert different regulatory effects on the amygdala. In human, the dorsal portion of the ACC positively modulates the amygdala whereas the ventral portion of the ACC exerts an inverse effect on the amygdala (Yamasaki et al., 2002, Das et al., 2005). Breakdown of this dynamic interplay between ACC and amygdala has been postulated to give rise to a range of neuropsychiatric disorders (Cremers et al., 2010, Lu et al., 2012, Schultz et al., 2012). Clinical functional imaging studies have demonstrated altered ACC-amygdala coupling in patients with PTSD from both civilian and combat veteran population (Rauch et al., 2006, Fonzo et al., 2010, Nardo et al., 2010, Yin et al., 2011, Sripada et al., 2012). One of the limitations of the current study is that the possible disruption of ACC-amygdala coupling was not addressed. Our findings of hyperactivity in both amygdala and ACC of blasted rats imply that altered ACC-amygdala coupling is possible, but it is unlikely that the net effect of ACC on amygdala for the blasted rats is negative modulation.

*Roles of the nucleus accumbens and hippocampus in tinnitus-related stress*

Previous studies have indicated the participation of the nucleus accumbens and hippocampus in the mechanism of tinnitus or PTSD (Karl et al., 2006, Werner et al., 2009, Rauschecker et al., 2010, Schecklmann et al., 2013). Our results of $Mn^{2+}$ uptake did not reflect this phenomenon, partly due to the complexity of the pathophysiology of the blast-induced

neurotrauma. Blast-induced traumatic brain injury has profound and diffuse impacts on all brain structures (Svetlov et al., 2009). Blast-induced tinnitus and its associated PTSD may thus have different pathological characteristics from tinnitus or PTSD rooted from other causes. In a combined behavioral, proteomics and histological study utilizing a stressed rat model, Kwon and colleagues compared the differences between blasted and stressed rats and the stressed-only rats. The one-time blast was delivered at 20-psi overpressure, and 2-week chronic stress was delivered with stressors such as fox urine, loud noises, and cage movements. The results showed that blasted and stressed rats exhibited lasting neuronal and glial cell loss, inflammation and gliosis in pre-frontal cortex and hippocampus whereas stressed-only rats did not (Kwon et al., 2011). The interplay of neuronal apoptosis and synaptic plasticity renders the uncertainty of the direction of the net result of $Mn^{2+}$ uptake in hippocampus and other paralimbic structures such as the nucleus accumbens. Our findings from the Morris water maze testing indicated that blast-induced tinnitus and related PTSD-like symptoms did not affect spatial memory; this finding resonates with previous studies of intense noise-induced tinnitus (Zheng et al., 2011)(Pace and Zhang, 2013).

**Conclusions**

The current study using combined behavioral assays and MEMRI to investigate the mechanisms of limbic involvement in blast-induced tinnitus and related PTSD-like symptoms. Our results indicated that 1) more than half of the blasted rats retained chronic tinnitus; 2) the majority of blasted rats exhibited high anxiety levels, and; 3) rats with blast-induced tinnitus showed hyperactivity in the amygdala, indicating that the amygdala is the key structure underpinning both tinnitus and post-traumatic stress. In addition, blasted rats showed hyperactivity in the anterior cingulate cortex, indicating that both hearing impairment and

traumatic brain injury plays a direct role in the induced changes. Spatial memory was not affected by the blast. We postulate that the amygdala participates in the pathogenesis of chronic tinnitus induced by blast through the thalamo-cortico-amygdala pathway, and that the anterior cingulate cortex is involved in the mechanisms of the blast-induced tinnitus post-traumatic stress, likely via alteration of ACC-amygdala coupling.

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## References

Aikins DE, Jackson ED, Christensen A, Walderhaug E, Afroz S, Neumeister A (2011) Differential conditioned fear response predicts duloxetine treatment outcome in male veterans with PTSD: a pilot study. Psychiatry research 188:453-455.

Bass CR, Panzer MB, Rafaels KA, Wood G, Shridharani J, Capehart B (2012) Brain injuries from blast. Annals of biomedical engineering 40:185-202.

Bissig D, Berkowitz BA (2009) Manganese-enhanced MRI of layer-specific activity in the visual cortex from awake and free-moving rats. Neuroimage 44:627-635.

Brennan FX, Beck KD, Ross RJ, Servatius RJ (2005) Stress-induced increases in avoidance responding: an animal model of post-traumatic stress disorder behavior? Neuropsychiatric disease and treatment 1:69-72.

Bush G, Luu P, Posner MI (2000) Cognitive and emotional influences in anterior cingulate cortex. Trends in cognitive sciences 4:215-222.

Chen J, Cui X, Zacharek A, Cui Y, Roberts C, Chopp M (2011) White matter damage and the effect of matrix metalloproteinases in type 2 diabetic mice after stroke. Stroke 42:445-452.

Corley MJ, Caruso MJ, Takahashi LK (2012) Stress-induced enhancement of fear conditioning and sensitization facilitates extinction-resistant and habituation-resistant fear behaviors in a novel animal model of posttraumatic stress disorder. Physiology & behavior 105:408-416.

Cremers HR, Demenescu LR, Aleman A, Renken R, van Tol MJ, van der Wee NJ, Veltman DJ, Roelofs K (2010) Neuroticism modulates amygdala-prefrontal connectivity in response to negative emotional facial expressions. NeuroImage 49:963-970.

Crossgrove JS, Yokel RA (2005) Manganese distribution across the blood-brain barrier. IV. Evidence for brain influx through store-operated calcium channels. Neurotoxicology 26:297-307.

Darley DS, Kellman RM (2010) Otologic considerations of blast injury. Disaster medicine and public health preparedness 4:145-152.

Das P, Kemp AH, Liddell BJ, Brown KJ, Olivieri G, Peduto A, Gordon E, Williams LM (2005) Pathways for fear perception: modulation of amygdala activity by thalamo-cortical systems. NeuroImage 26:141-148.

De Ridder D, Fransen H, Francois O, Sunaert S, Kovacs S, Van De Heyning P (2006) Amygdalohippocampal involvement in tinnitus and auditory memory. Acta oto-laryngologica Supplementum 50-53.

Elder GA, Dorr NP, De Gasperi R, Gama Sosa MA, Shaughness MC, Maudlin-Jeronimo E, Hall AA, McCarron RM, Ahlers ST (2012) Blast exposure induces post-traumatic stress disorder-related traits in a rat model of mild traumatic brain injury. Journal of neurotrauma 29:2564-2575.

Etkin A, Egner T, Peraza DM, Kandel ER, Hirsch J (2006) Resolving emotional conflict: a role for the rostral anterior cingulate cortex in modulating activity in the amygdala. Neuron 51:871-882.

Fagelson MA (2007) The association between tinnitus and posttraumatic stress disorder. American journal of audiology 16:107-117.

Feng J, Bendiske J, Morest DK (2012) Degeneration in the ventral cochlear nucleus after severe noise damage in mice. J Neurosci Res 90:831-841.

Fitch RH, Threlkeld SW, McClure MM, Peiffer AM (2008) Use of a modified prepulse inhibition paradigm to assess complex auditory discrimination in rodents. Brain research bulletin 76:1-7.

Fonzo GA, Simmons AN, Thorp SR, Norman SB, Paulus MP, Stein MB (2010) Exaggerated and disconnected insular-amygdalar blood oxygenation level-dependent response to threat-related emotional faces in women with intimate-partner violence posttraumatic stress disorder. Biol Psychiatry 68:433-441.

Goble TJ, Moller AR, Thompson LT (2009) Acute high-intensity sound exposure alters responses of place cells in hippocampus. Hear Res 253:52-59.

Hinton DE, Chhean D, Pich V, Hofmann SG, Barlow DH (2006) Tinnitus among Cambodian refugees: relationship to PTSD severity. Journal of traumatic stress 19:541-546.

Hoare DJ, Hall DA (2011) Clinical guidelines and practice: a commentary on the complexity of tinnitus management. Evaluation & the health professions 34:413-420.

Hoare DJ, Kowalkowski VL, Kang S, Hall DA (2011) Systematic review and meta-analyses of randomized controlled trials examining tinnitus management. Laryngoscope 121:1555-1564.

Jehel L, Paterniti S, Brunet A, Duchet C, Guelfi JD (2003) Prediction of the occurrence and intensity of post-traumatic stress disorder in victims 32 months after bomb attack. European psychiatry : the journal of the Association of European Psychiatrists 18:172-176.

Kaltenbach JA, Zhang J (2007) Intense sound-induced plasticity in the dorsal cochlear nucleus of rats: evidence for cholinergic receptor upregulation. HearRes 226:232-243.

Karl A, Schaefer M, Malta LS, Dorfel D, Rohleder N, Werner A (2006) A meta-analysis of structural brain abnormalities in PTSD. Neuroscience and biobehavioral reviews 30:1004-1031.

Kraus KS, Canlon B (2012) Neuronal connectivity and interactions between the auditory and limbic systems. Effects of noise and tinnitus. Hearing research 288:34-46.

Kraus KS, Ding D, Jiang H, Lobarinas E, Sun W, Salvi RJ (2011) Relationship between noise-induced hearing-loss, persistent tinnitus and growth-associated protein-43 expression in the rat cochlear nucleus: does synaptic plasticity in ventral cochlear nucleus suppress tinnitus? Neuroscience 194:309-325.

Kwon SK, Kovesdi E, Gyorgy AB, Wingo D, Kamnaksh A, Walker J, Long JB, Agoston DV (2011) Stress and traumatic brain injury: a behavioral, proteomics, and histological study. Frontiers in neurology 2:12.

Landgrebe M, Langguth B, Rosengarth K, Braun S, Koch A, Kleinjung T, May A, de Ridder D, Hajak G (2009) Structural brain changes in tinnitus: grey matter decrease in auditory and non-auditory brain areas. NeuroImage 46:213-218.

Lazar SW, Bush G, Gollub RL, Fricchione GL, Khalsa G, Benson H (2000) Functional brain mapping of the relaxation response and meditation. Neuroreport 11:1581-1585.

Leaver AM, Renier L, Chevillet MA, Morgan S, Kim HJ, Rauschecker JP (2011) Dysregulation of limbic and auditory networks in tinnitus. Neuron 69:33-43.

Lee JH, Silva AC, Merkle H, Koretsky AP (2005) Manganese-enhanced magnetic resonance imaging of mouse brain after systemic administration of MnCl2: dose-dependent and temporal evolution of T1 contrast. Magn Reson Med 53:640-648.

Leonardi AD, Bir CA, Ritzel DV, VandeVord PJ (2011) Intracranial pressure increases during exposure to a shock wave. Journal of neurotrauma 28:85-94.

Lew HL, Jerger JF, Guillory SB, Henry JA (2007) Auditory dysfunction in traumatic brain injury. J Rehabil Res Dev 44:921-928.

Liu XS, Chopp M, Kassis H, Jia LF, Hozeska-Solgot A, Zhang RL, Chen C, Cui YS, Zhang ZG (2012) Valproic acid increases white matter repair and neurogenesis after stroke. Neuroscience 220:313-321.

Lockwood AH, Salvi RJ, Coad ML, Towsley ML, Wack DS, Murphy BW (1998) The functional neuroanatomy of tinnitus: evidence for limbic system links and neural plasticity. Neurology 50:114-120.

Longenecker RJ, Galazyuk AV (2011) Development of tinnitus in CBA/CaJ mice following sound exposure. J Assoc Res Otolaryngol 12:647-658.

Lu Q, Li H, Luo G, Wang Y, Tang H, Han L, Yao Z (2012) Impaired prefrontal-amygdala effective connectivity is responsible for the dysfunction of emotion process in major depressive disorder: a dynamic causal modeling study on MEG. Neuroscience letters 523:125-130.

Luo H, Zhang X, Nation J, Pace E, Lepczyk L, Zhang J (2012) Tinnitus suppression by electrical stimulation of the rat dorsal cochlear nucleus. Neurosci Lett.

Mahlke C, Wallhausser-Franke E (2004) Evidence for tinnitus-related plasticity in the auditory and limbic system, demonstrated by arg3.1 and c-fos immunocytochemistry. Hearing research 195:17-34.

Mao JC, Pace E, Pierozynski P, Kou Z, Shen Y, Vandevord P, Haacke EM, Zhang X, Zhang J (2012) Blast-induced tinnitus and hearing loss in rats: behavioral and imaging assays. J Neurotrauma 29:430-444.

Matsumoto Y, Morinobu S, Yamamoto S, Matsumoto T, Takei S, Fujita Y, Yamawaki S (2013) Vorinostat ameliorates impaired fear extinction possibly via the hippocampal NMDA-CaMKII pathway in an animal model of posttraumatic stress disorder. Psychopharmacology 229:51-62.

Mirz F, Gjedde A, Ishizu K, Pedersen CB (2000) Cortical networks subserving the perception of tinnitus--a PET study. Acta oto-laryngologica Supplementum 543:241-243.

Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. Journal of neuroscience methods 11:47-60.

Nardo D, Hogberg G, Looi JC, Larsson S, Hallstrom T, Pagani M (2010) Gray matter density in limbic and paralimbic cortices is associated with trauma load and EMDR outcome in PTSD patients. Journal of psychiatric research 44:477-485.

Nobre MJ, Brandao ML (2011) Modulation of auditory-evoked potentials recorded in the inferior colliculus by GABAergic mechanisms in the basolateral and central nuclei of the amygdala in high- and low-anxiety rats. Brain Res 1421:20-29.

North CS, Nixon SJ, Shariat S, Mallonee S, McMillen JC, Spitznagel EL, Smith EM (1999) Psychiatric disorders among survivors of the Oklahoma City bombing. JAMA : the journal of the American Medical Association 282:755-762.

Nowotny M, Remus M, Kossl M, Gaese BH (2011) Characterization of the perceived sound of trauma-induced tinnitus in gerbils. J Acoust Soc Am 130:2827-2834.

Owens BD, Kragh JF, Jr., Wenke JC, Macaitis J, Wade CE, Holcomb JB (2008) Combat wounds in operation Iraqi Freedom and operation Enduring Freedom. The Journal of trauma 64:295-299.

Perrine SA, Hoshaw BA, Unterwald EM (2006) Delta opioid receptor ligands modulate anxiety-like behaviors in the rat. British journal of pharmacology 147:864-872.

Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A (2000) Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. Annals of the New York Academy of Sciences 911:369-391.

Plewnia C, Reimold M, Najib A, Reischl G, Plontke SK, Gerloff C (2007) Moderate therapeutic efficacy of positron emission tomography-navigated repetitive transcranial magnetic stimulation for chronic tinnitus: a randomised, controlled pilot study. Journal of neurology, neurosurgery, and psychiatry 78:152-156.

Rauch SL, Shin LM, Phelps EA (2006) Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research--past, present, and future. Biol Psychiatry 60:376-382.

Rauschecker JP, Leaver AM, Muhlau M (2010) Tuning out the noise: limbic-auditory interactions in tinnitus. Neuron 66:819-826.

Risling M, Plantman S, Angeria M, Rostami E, Bellander BM, Kirkegaard M, Arborelius U, Davidsson J (2011) Mechanisms of blast induced brain injuries, experimental studies in rats. NeuroImage 54 Suppl 1:S89-97.

Romanski LM, LeDoux JE (1992) Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. The Journal of neuroscience : the official journal of the Society for Neuroscience 12:4501-4509.

Sah P, Lopez De Armentia M (2003) Excitatory synaptic transmission in the lateral and central amygdala. Annals of the New York Academy of Sciences 985:67-77.

Schecklmann M, Landgrebe M, Poeppl TB, Kreuzer P, Manner P, Marienhagen J, Wack DS, Kleinjung T, Hajak G, Langguth B (2013) Neural correlates of tinnitus duration and distress: a positron emission tomography study. Hum Brain Mapp 34:233-240.

Schultz DH, Balderston NL, Helmstetter FJ (2012) Resting-state connectivity of the amygdala is altered following Pavlovian fear conditioning. Frontiers in human neuroscience 6:242.

Shekhawat GS, Searchfield GD, Stinear CM (2013) The relationship between tinnitus pitch and hearing sensitivity. Eur Arch Otorhinolaryngol.

Shussman N, Mintz A, Zamir G, Shalev A, Gazala MA, Rivkind AI, Isenberg Y, Bala M, Almogy G (2011) Posttraumatic stress disorder in hospitalized terrorist bombing attack victims. The Journal of trauma 70:1546-1550.

Silva AC, Bock NA (2008) Manganese-enhanced MRI: an exceptional tool in translational neuroimaging. Schizophrenia bulletin 34:595-604.

Sripada RK, King AP, Garfinkel SN, Wang X, Sripada CS, Welsh RC, Liberzon I (2012) Altered resting-state amygdala functional connectivity in men with posttraumatic stress disorder. Journal of psychiatry & neuroscience : JPN 37:241-249.

Stutzmann GE, McEwen BS, LeDoux JE (1998) Serotonin modulation of sensory inputs to the lateral amygdala: dependency on corticosterone. The Journal of neuroscience : the official journal of the Society for Neuroscience 18:9529-9538.

Svetlov SI, Larner SF, Kirk DR, Atkinson J, Hayes RL, Wang KK (2009) Biomarkers of blast-induced neurotrauma: profiling molecular and cellular mechanisms of blast brain injury. Journal of neurotrauma 26:913-921.

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, Hughes LF, Caspary DM (2006a) Gap detection deficits in rats with tinnitus: a potential novel screening tool. BehavNeurosci 120:188-195.

Turner JG, Brozoski TJ, Bauer CA, Parrish JL, Myers K, Hughes LF, Caspary DM (2006b) Gap detection deficits in rats with tinnitus: a potential novel screening tool. Behavioral neuroscience 120:188-195.

Ueno Y, Chopp M, Zhang L, Buller B, Liu Z, Lehman NL, Liu XS, Zhang Y, Roberts C, Zhang ZG (2012) Axonal outgrowth and dendritic plasticity in the cortical peri-infarct area after experimental stroke. Stroke 43:2221-2228.

van Wingen GA, Geuze E, Vermetten E, Fernandez G (2011) Perceived threat predicts the neural sequelae of combat stress. Molecular psychiatry 16:664-671.

VBA (2013) VA Annual Benefits Report: Fiscal Year 2012. vol. 1 (VA, U. S., ed), p 82 810 Vermont Avenue N. W. , Washington, D.C., 20420: Veterans Benefits Administration.

Wallhausser-Franke E, Mahlke C, Oliva R, Braun S, Wenz G, Langner G (2003) Expression of c-fos in auditory and non-auditory brain regions of the gerbil after manipulations that induce tinnitus. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 153:649-654.

Wang H, Brozoski TJ, Turner JG, Ling L, Parrish JL, Hughes LF, Caspary DM (2009) Plasticity at glycinergic synapses in dorsal cochlear nucleus of rats with behavioral evidence of tinnitus. Neuroscience 164:747-759.

Wang H, Zuo D, He B, Qiao F, Zhao M, Wu Y (2012) Conditioned fear stress combined with single-prolonged stress: a new PTSD mouse model. Neuroscience research 73:142-152.

Werner NS, Meindl T, Engel RR, Rosner R, Riedel M, Reiser M, Fast K (2009) Hippocampal function during associative learning in patients with posttraumatic stress disorder. Journal of psychiatric research 43:309-318.

Yamasaki H, LaBar KS, McCarthy G (2002) Dissociable prefrontal brain systems for attention and emotion. Proceedings of the National Academy of Sciences of the United States of America 99:11447-11451.

Yang G, Lobarinas E, Zhang L, Turner J, Stolzberg D, Salvi R, Sun W (2007) Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. HearRes 226:244-253.

Yang S, Su W, Bao S (2012) Long-term, but not transient, threshold shifts alter the morphology and increase the excitability of cortical pyramidal neurons. J Neurophysiol 108:1567-1574.

Yin Y, Jin C, Hu X, Duan L, Li Z, Song M, Chen H, Feng B, Jiang T, Jin H, Wong C, Gong Q, Li L (2011) Altered resting-state functional connectivity of thalamus in earthquake-

induced posttraumatic stress disorder: a functional magnetic resonance imaging study. Brain research 1411:98-107.

Zhang J (2013) Auditory cortex stimulation to suppress tinnitus: Mechanisms and strategies. Hear Res 295:38-57.

Zhang J, Guan Z (2008) Modulatory effects of somatosensory electrical stimulation on neural activity of the dorsal cochlear nucleus of hamsters. JNeurosciRes 86:1178-1187.

Zhang J, Zhang Y, Zhang X (2011) Auditory cortex electrical stimulation suppresses tinnitus in rats. J Assoc Res Otolaryngol 12:185-201.

Zhang JS, Kaltenbach JA, Wang J, Kim SA (2003) Fos-like immunoreactivity in auditory and nonauditory brain structures of hamsters previously exposed to intense sound. Exp Brain Res 153:655-660.

Zhang Y, Chopp M, Mahmood A, Meng Y, Qu C, Xiong Y (2012) Impact of inhibition of erythropoietin treatment-mediated neurogenesis in the dentate gyrus of the hippocampus on restoration of spatial learning after traumatic brain injury. Exp Neurol 235:336-344.

Zheng Y, Hamilton E, Begum S, Smith PF, Darlington CL (2011) The effects of acoustic trauma that can cause tinnitus on spatial performance in rats. Neuroscience 186:48-56.

Zovkic IB, Sweatt JD (2013) Epigenetic mechanisms in learned fear: implications for PTSD. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 38:77-93.

Zucker RS, Smith SJ (1979) Effect of TEA on light emission from aequorin-injected aplysia central neurons. Brain Res 169:91-102.